



**RADIATION BIOLOGY**  
***VOLUME II***

(315)



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# RADIATION BIOLOGY

VOLUME II: ULTRAVIOLET AND RELATED RADIATIONS

*Edited by*

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## RADIATION BIOLOGY, VOLUME II

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This volume is dedicated to the memory of  
two members of the Volume II Editorial Committee

DR. LEWIS J. STADLER

Professor of Field Crops, University of Missouri  
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and

DR. JOHN R. LOOFBOUROW

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## PREFACE

This second volume of "Radiation Biology" covers the field of ultraviolet radiation. It also contains considerable material on high-energy radiation. Since both parts of the spectrum induce similar biological effects, no clear line can be drawn between the two. Microbiology, for example, is discussed in both volumes but occupies a more prominent place in the second. Actually, Volume I and Volume II compose a unit and should be read as such.

ALEXANDER HOLLAENDER



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# RADIATION BIOLOGY

*Volume II*



## CHAPTER 1

# Photochemistry

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*Introduction. Primary steps: Absorption—Franck-Condon principle—Direct optical dissociation—Half life of the excited state—Predissociation—Internal conversion—Phosphorescence and long-lived fluorescence—Long-lived energetic states—Quenching of excited states—Transfer of excitation—Solvent effects—Cage effect—Photochemical transfer of electrons or protons to the solvent. Secondary steps: Bimolecular steps—Unimolecular steps—Termolecular steps—Diffusion-controlled processes. Mechanism of complex reactions: General problem—Steady-state approximation. Examples of the principal types of photochemical reactions: Decomposition reactions—Reactions of molecular oxygen—Polymerization and dimerization—Intramolecular changes—Sensitized reactions. References.*

## INTRODUCTION

The development of photochemistry as a quantitative science was made possible by the discovery of the Einstein photochemical equivalence law and by the accumulation of spectroscopic knowledge. The equivalence law can be stated as follows: a photon can induce a photochemical reaction only by being absorbed and, on being absorbed, will activate one and only one molecule. The spectroscopic behavior of practically all (stable) diatomic molecules is well understood. In studying the optical properties of complex molecules, it is usually necessary to be guided by general principles and qualitative analogies. In principle, there is little difference between photochemical reactions utilizing visible light and those produced by ultraviolet radiation.

The observed change in a photochemical reaction, as in a thermal reaction, is the result of the concurrence of a number of simple reaction steps. A set of reaction steps, which is consistent with all available information about a reaction, is called the "mechanism of the reaction." It is convenient to divide the steps which constitute the mechanism into two groups, called "primary" and "secondary" steps. The primary steps are those chemical or physical processes which are the direct consequence of the absorption of the photon and which involve only the absorbing species (and possibly the solvent). Reactive molecules, radicals, or atoms are produced by the primary steps. These reactive entities can

then undergo (or initiate) a series of simple thermal reactions, the secondary steps. Secondary reaction steps are unimolecular, bimolecular, or termolecular reactions. Of these, bimolecular reactions, which involve a radical or atom and a stable molecule, are probably the most important. Bimolecular reactions also occur between two radicals or atoms. In the gas phase, recombination of two atoms always occurs as the result of a three-body collision. This is very probably the only important type of termolecular reaction step. Unimolecular reaction steps are limited to the "spontaneous" decomposition or rearrangement of complex molecules.

## PRIMARY STEPS

### ABSORPTION

Each photon is absorbed by a single molecule,<sup>1</sup> and, under all ordinary conditions, multiple excitation of a molecule by successive capture of two or more photons is of negligible importance. By capture of a photon the molecule is raised from the ground state to a higher electronic state. With few exceptions (notably molecular oxygen and "odd" molecules; Herzberg, 1950), the ground state of a stable molecule is a singlet one, and the molecule will be excited preferentially to a higher singlet level. The selection rule, which "forbids" transitions between states of different multiplicities, is called the "intercombination rule." For ordinary molecules the probability of a transition between energy levels of unlike multiplicity can be  $10^6$ -fold less than that for an otherwise similar transition which does not violate the rule. This is one of the few selection rules which apply to complex molecules as well as to atoms and diatomic molecules. For certain cases, such as those involving heavy atoms, e.g., mercury, the rule applies less strictly, and the probability of transition is reduced by a factor of only about  $10^3$ .

### FRANCK-CONDON PRINCIPLE

The Franck-Condon principle is probably of greater importance than the selection rules in determining the photochemical behavior of molecules. This principle states that electronic transitions which involve appreciable changes in the positions or momenta of the constituent nuclei have relatively low probabilities.<sup>2</sup> At ordinary temperatures, molecules which exist in thermal equilibrium with their surroundings are in their lowest oscillational states. Accordingly, the Franck-Condon principle and the potential-energy curves determine the oscillational

<sup>1</sup> In this discussion, certain types of crystals and crystalline micelles are to be regarded as single molecules.

<sup>2</sup> This discussion of the Franck-Condon principle is based on its original classical formulation. For a discussion of the modifications introduced by quantum theory see Herzberg (1950).



states of the excited electronic state that can be reached directly by the absorption of a photon. This is illustrated for a diatomic molecule in Fig. 1-1, in which the ordinate  $r$  represents the distance between the nuclei and  $U$  is the potential energy of the molecule. In Fig. 1-1a, the equilibrium separation for the nuclei is about the same in the excited state as it is in the ground state. The probable transitions from the ground state can be represented by vertical arrows lying between  $a$  and  $b$  since they correspond to small changes of position or momentum. In this way, a simple application of the Franck-Condon principle demonstrates that direct optical dissociation is improbable for a molecule whose potential-energy curves are of the type illustrated in Fig. 1-1a. In the gas phase at low pressure, where the life expectancy of an excited molecule is short compared to the time interval between collisions, such a molecule would emit one quantum for each one absorbed. As is suggested by the variations in the length of the downward arrows, the fluorescence or emission spectrum is much more complex than the corresponding absorption spectrum. In a condensed phase or in a gas at higher pressure, there is a high probability of the excited molecule losing its excess oscillational energy by collisions of the second kind (Franck, 1922) during the lifetime of the excited state. As a result, practically all the transitions, corresponding to the fluorescence, will start from the lowest vibrational level of the excited state. Stokes's law holds under these conditions, and the fluorescence spectrum is shifted to the red side of the absorption spectrum. Frequently the fluorescence spectrum is approximately a mirror image of the absorption spectrum (Lewschin, 1931).

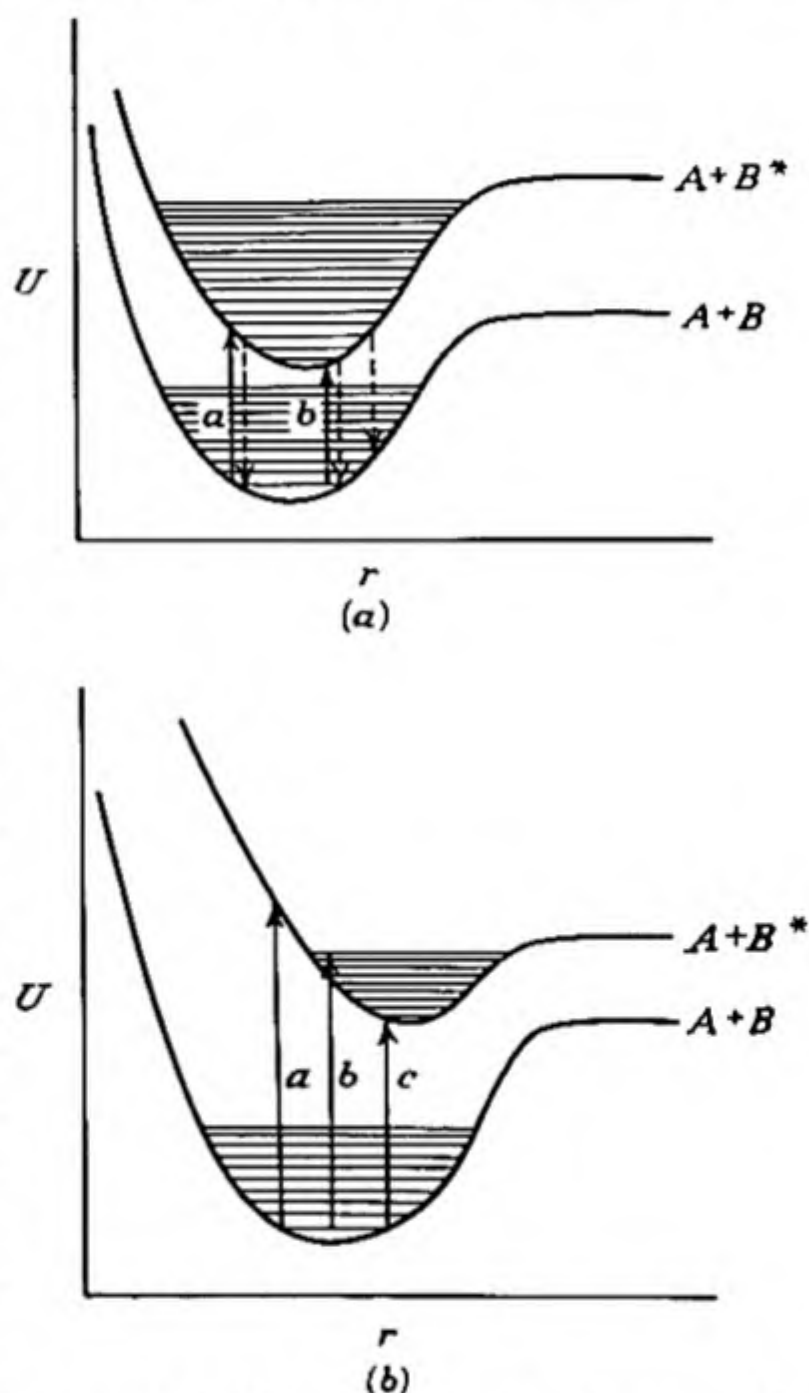


FIG. 1-1. Potential-energy diagrams for a diatomic molecule.

### DIRECT OPTICAL DISSOCIATION

In the case illustrated by Fig. 1-1b, the molecule is much less stable in its excited than in its ground state and, correspondingly, the equilibrium separation of the nuclei is increased. It follows, from the Franck-Condon

principle, that the range of probable transitions (starting from the ground state) lies between the vertical arrows  $a$  and  $c$ . Transitions whose arrows lie between arrows  $b$  and  $c$  are similar to those discussed in connection with Fig. 1-1a. Transitions arising from points to the left of arrow  $b$  result in the formation of an excited molecule whose vibrational energy is greater than that required to dissociate it into atoms, one normal and one excited. Accordingly, direct optical dissociation is a probable event. If the gas is irradiated with photons of energy greater than that indicated by the length of arrow  $b$  and less than that of arrow  $a$ , the radiation will be strongly absorbed. For each such absorption act, a molecule will be dissociated (within the half period of a single vibration), and there will be no fluorescent emission. Since the end state is not quantized, the absorption spectrum will be continuous in this region.

### HALF LIFE OF THE EXCITED STATE

If no chemical process, such as optical dissociation, can take place, the half life ( $\tau_{1/2}$ ) of the excited state of a molecule is greater than  $10^{-9}$  sec, and the molecule will lose all or part of its energy of excitation by emitting a photon. Since the restrictions which limit the probability of a transition apply equally to absorption and emission, a high absorption coefficient corresponds to a short half life of the excited state. For example, the direct optical excitation of a normal molecule from its ground singlet state to an excited triplet state (with a half life of  $10^{-3}$  sec) would be so improbable that it would not be observed in the absorption spectrum under ordinary conditions. These conditions were put in quantitative form by Einstein in 1917 (Herzberg, 1950, pp. 20, 381). If the effect of possible degeneracy of the levels is neglected, the Einstein relation can be put in the following form:

$$\alpha\tau_{1/2} = \frac{N_A c \ln 2}{8\pi \times 10^3} \times \frac{1}{\nu^2}, \quad (1-1)$$

where  $N_A$  is Avogadro's number,  $c$  is the velocity of light, and  $\nu$  is the frequency of the radiation absorbed or emitted. The Beer's law extinction coefficient,  $\alpha$ , is defined by the following equation, in which  $m$  is the concentration of the absorbent in moles per liter,  $l$  is the length of the light path in centimeters and  $I_0$  and  $I_{tr}$  are the intensities, respectively, of the incident and transmitted light:

$$I_{tr} = I_0 e^{-\alpha m l}.$$

It should be noted that the product  $\alpha\tau_{1/2}$  is inversely proportional to the square of the frequency. While Eq. (1-1) applies only to monochromatic light (and therefore approximately to atomic spectra), it can be modified to apply to the broad-band absorption and emission of a molecule. For the latter application,  $\alpha$  must be known as an empirical function of  $\nu$ , and



the expression must be integrated over all frequencies which correspond to the electronic transition under consideration.

Equation (1-1) is based on the assumption that an excited molecule can lose its energy of excitation only by emitting a photon. If the energy can be lost in any other way, either spontaneous or induced, the lifetime of the excited state will be correspondingly reduced. When the system is illuminated with light of constant intensity, a steady-state condition will prevail, and the concentration  $N_N$  of molecules in the excited state will be constant. If "intensities" are expressed in photons absorbed per cubic centimeter per second and the rate  $\nu_c$  of the nonradiative disappearance of excited molecules in corresponding units, then

$$I_{abs} = I_{fl} + \nu_c = (k_{fl} + k_c)N_N.$$

The coefficient  $k_c$  can be a function of added substances, but, for any given solution,  $k_{fl} + k_c$  is a constant and is equal to the reciprocal of the mean life  $\tau$  of the excited state under these special conditions. Since

$$I_{fl} = k_{fl}N_N,$$

it follows that

$$\frac{I_{fl}}{I_{abs}} = \frac{k_{fl}}{k_{fl} + k_c} = \frac{\tau}{\tau_0}, \quad (1-2)$$

where  $\tau_0$  is the natural mean life or the life which the excited state would have if the emission of fluorescent light was the only possible degradative process. The ratio of  $I_{fl}/I_{abs}$  is called the fluorescence yield.

### PREDISSOCIATION

The fluorescence yield, and correspondingly the mean life, of an excited molecule may be reduced by a process known as "predissociation." This process is possible when a stable vibrational level of an electronically excited state overlaps a dissociation region of another state. For a diatomic molecule this corresponds to the crossing of the potential-energy curves of two excited states. Under these conditions, when both the energy and the nuclear configuration are the same in the two states, the molecules can cross over from the stable, quantized state into the unstable, nonquantized state. While the energy of the primary excited state must be greater than the thermochemical energy of dissociation, it may be much less than that required for direct optical dissociation. The probability of such a transition may have any value from unity to practically zero. It is determined by the Franck-Condon principle and by certain selection rules. The chemical detection of the resultant atoms or radicals is the most sensitive test for the occurrence of predissociation. The weakening of the fluorescence intensity is a direct measure of the probability of predissociation. The disappearance of the rotational structure of an absorption band indicates that the probability of the cross-



over from one state to the other has become so great that the mean life of the excited molecule has been reduced to a value comparable to its period of rotation.

Some molecules which exhibit a fluorescence yield of unity at low pressures dissociate when they are illuminated at high pressures. A collision of the excited molecule with a normal molecule of its own kind or of an added gas induces its dissociation. A process of this type, which is called "induced predissociation," was first observed for  $I_2$  (Herzberg, 1950).

### INTERNAL CONVERSION

In addition to fluorescence, direct optical dissociation, and predissociation, excited complex molecules can undergo a process called "internal conversion" (Franck and Sponer, 1949). This process consists in a radiationless transition from a low oscillational level of a higher electronic state to a high oscillational level of a lower electronic state. The difference in energy between the two electronic states appears as an increase in the oscillational energy of the molecule after the transition. In internal conversion, both the initial and final states are quantized; in this respect internal conversion differs from predissociation. Like predissociation, it can occur only when the molecule is in a specific nuclear configuration for which the total energy and the nuclear configuration of the molecule are the same in the two electronic states. Since a complex molecule has many generalized oscillational degrees of freedom, the time required for the molecule to reach the required configuration may be relatively long. Under experimentally realizable conditions, the time between collisions appears to be much less than the average time required for the molecule in the lower electronic state to return to the crossing point and thus to have a chance of coming back to the original state. Collisions between surrounding molecules and the vibrationally excited molecule quickly reduce the vibrational energy of the latter and so make the reverse transition impossible. In this way, internal conversion followed by a number of collisions of the second kind can lead to the complete degradation of the energy of excitation into thermal energy of the system. This is very probably the explanation of why many molecules which absorb strongly in the visible or near ultraviolet are nonfluorescent and do not react photochemically.

Immediately after the act of internal conversion, the molecule has a large amount of energy in its oscillational degrees of freedom; in other words, it is a "hot" molecule. As such it can undergo pyrolytic reactions such as decarboxylation or the elimination of a hydrogen molecule. It is difficult to conceive of any other simple explanation of the direct photochemical dissociation of a complex molecule into two stable molecules, a process which requires the simultaneous breaking of several bonds and the formation of new bonds. Depending on the nature of the molecule and

the amount of energy available, internal conversion may lead to the dissociation of a molecule either into two stable molecules or into radicals.

### PHOSPHORESCENCE AND LONG-LIVED FLUORESCENCE

Metastable states appear to play an important part in the photochemistry of complex molecules. The existence of these states has been demonstrated indirectly by the analysis of photochemical data (Shpol'skiĭ and Sheremet'ev, 1936) and directly by a study of the "phosphorescence" and "long-lived fluorescence" of these molecules (Pringsheim, 1949; Förster, 1951). Practically all complex molecules (at least those which contain a double bond) are either fluorescent or phosphorescent (or both) when they are dissolved in glassy media or adsorbed on suitable solids. One of the first examples to be studied quantitatively was the dye tryptophan adsorbed on silica gel (Pringsheim and Vogels, 1936). At ordinary temperatures this system emits a strong fluorescent green band and a separate weak orange band. The green band is made up of ordinary short-lived fluorescence and a relatively long-lived emission, having the same wave-length distribution. The half life  $\tau_{1/2}$  corresponding to the latter process is an exponential function,  $\tau_{1/2} = ke^{E/RT}$ , of temperature. There is no short-lived fluorescence corresponding to the orange band. The half life corresponding to this latter transition is independent of temperature and equal to 1.2 sec. As the temperature is reduced, the life corresponding to the green phosphorescence eventually becomes longer than that pertaining to the orange, long-lived fluorescence, and the slow emission becomes predominantly orange. This general behavior is exhibited by a wide variety of substances (Kasha, 1947). Many measurements of this type (Lewis and Kasha, 1944, 1945) have been made with absorbing substance dissolved in a solvent, such as a mixture of ether, pentane, and alcohol, which is fluid at ordinary temperatures and becomes rigid at low temperatures. Under these conditions, only ordinary fluorescence is observed in the fluid solvent, the temperature-dependent phosphorescence appears when the solvent becomes very viscous, and the temperature-independent, long-lived fluorescence becomes noticeable at still lower temperatures.

A reasonable explanation of these phenomena, which was first proposed by Jablonski (1935), is illustrated by the simplified energy diagram of Fig. 1-2. The several electronic-energy levels are represented by horizontal lines, capped by a bundle of horizontal lines which indicate the overlapping generalized oscillational levels. For an ordinary stable molecule, the ground level  $N$  and the two excited levels  $F$  and  $F'$  are singlet states. The metastable level  $M$  is, presumably, a triplet level. The transitions which correspond to arrows 1 and 2 represent the absorption of photons, which raise the molecule into its first or second excited (singlet) state. In its initial state the molecule will be in thermal equilibrium with its surround-



ings, and its oscillational energy will be at or near its zero-point value. As determined by the Franck-Condon principle, the electronically excited molecule will usually have an excess of oscillational energy. In a condensed medium or in a gas at moderate pressure, the molecule will quickly lose this excess of vibrational energy by successive collisions of the second kind. As a result the fluorescent light, transition 3, will usually have a larger wave length than the corresponding absorption. In addition to these permitted transitions, radiationless transitions, 4, 5, and 6, are possible. Each of these acts corresponds to a process of internal conversion. The occurrence of step 4 is proved by the fact that fluorescence corresponding to transitions from  $F'$  to  $N$  or from  $F'$  to  $F$  is never observed

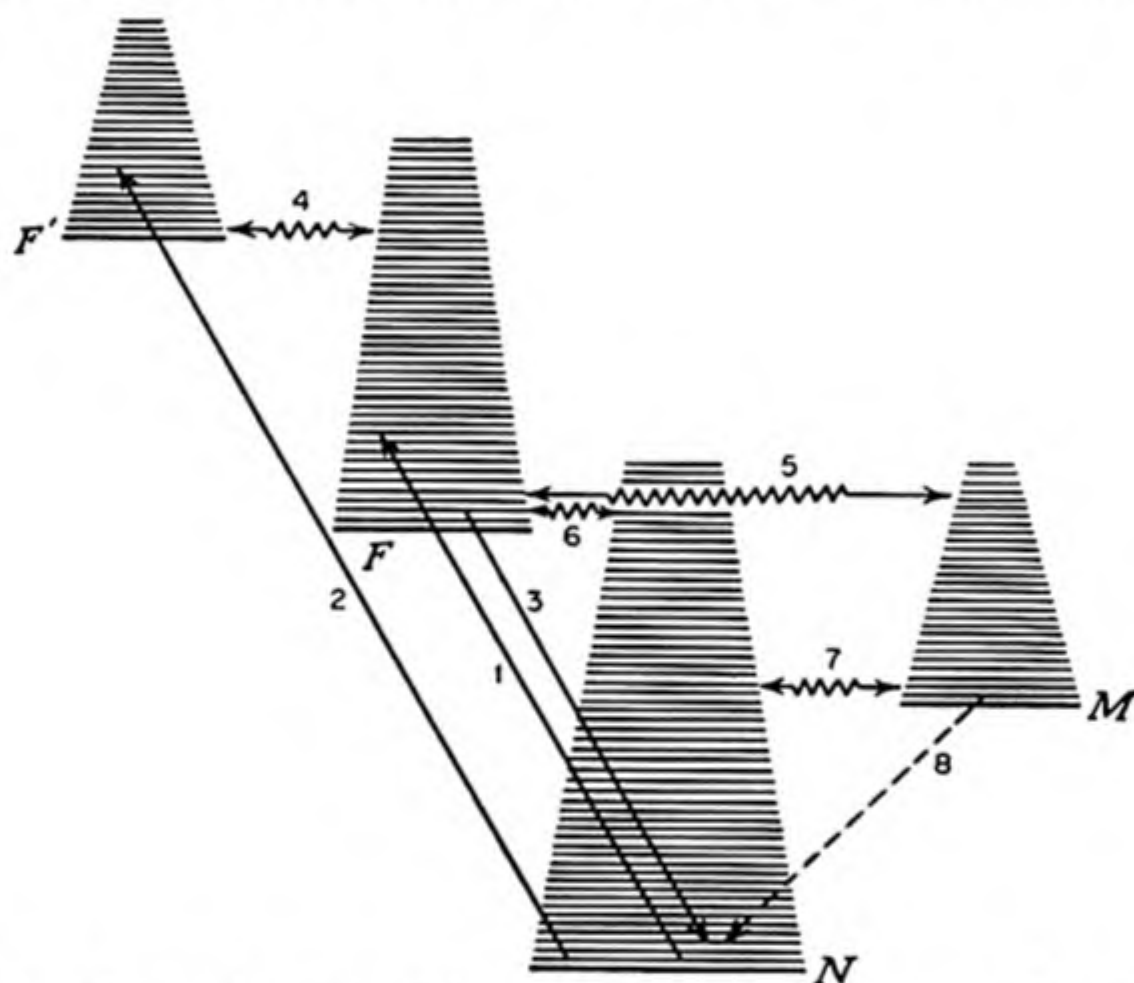


FIG. 1-2. Schematic potential-energy diagram for a complex molecule.

with complex molecules such as dyes. Illumination with light of shorter wave length, which raises the molecule to the second (or a higher excited) level  $F'$ , results only in the long-wave-length fluorescence (transition 3). Phosphorescence and long-lived fluorescence involve the metastable state  $M$ . In order to reach this state the molecule must undergo an act of internal conversion, step 5. Once in state  $M$ , the molecule can return to state  $F$ , by way of step 5, only if it receives thermal energy equal to or greater than  $\epsilon$ , the difference between the energies of levels  $M$  and  $F$ . This sequence of events corresponds to the temperature-dependent phosphorescence. Step 8 is forbidden, by the intercombination rule, and will occur only if there is no other probable mode of escape from  $M$ . The relative importance of this emission, the long-lived fluorescence, increases as the temperature is lowered. Internal-conversion steps, 6 or 7, contribute to the inefficiency of photochemical and fluorescence processes. One effect of adsorbing the molecule or dissolving it in a rigid medium is to

reduce its number of oscillational degrees of freedom and thereby to lower the probability of internal conversion. It seems reasonable to assume that this effect of binding the molecule to its surroundings has a relatively small effect on the initial transition from  $F$  to  $M$  (or  $F'$  to  $F$ ), since the molecule and its surroundings momentarily have available an energy excess equal to the difference between the energy of the photon and the energy difference between levels  $F$  and  $N$ .

If the preceding simple explanation is correct, the difference between the energy levels  $F$  and  $M$  can be determined in two independent ways, which should yield identical results. The difference in potential energy between levels  $F$  and  $N$  is measured approximately by the quantity  $h\nu_3$ , corresponding to the long-wave-length limit of the normal fluorescence. Similarly, the energy difference between  $M$  and  $N$  is approximately equal to  $h\nu_8$  for the long-wave-length limit of the long-lived fluorescence. The energy difference between  $F$  and  $M$  is therefore equal to  $h\nu_3 - h\nu_8$ . This same energy difference should be obtainable from measurements, at two or more temperatures, of the half life of the phosphorescence. In terms of the simple Jablonski mechanism,  $\epsilon$  in the following equation should be equal to  $h\nu_3 - h\nu_8$ :

$$\tau_{1/2} = ke^{\epsilon/RT}.$$

For all cases for which the experimental evidence is available, this is approximately true. Although there are minor and understandable discrepancies (Pringsheim, 1949, p. 441), the available data support this interpretation. The measured values (Kasha, 1947) of the  $M$ - $F$  energy difference lie in the range 5–40 kcal/mole. The lower values belong to dyes, and the higher ones to simpler molecules, such as the aromatic hydrocarbons. Since  $e^{-\epsilon/RT}$  will be a very small fraction for the higher values of  $\epsilon$ , phosphorescence will be very inefficient and probably undetectable in these cases.

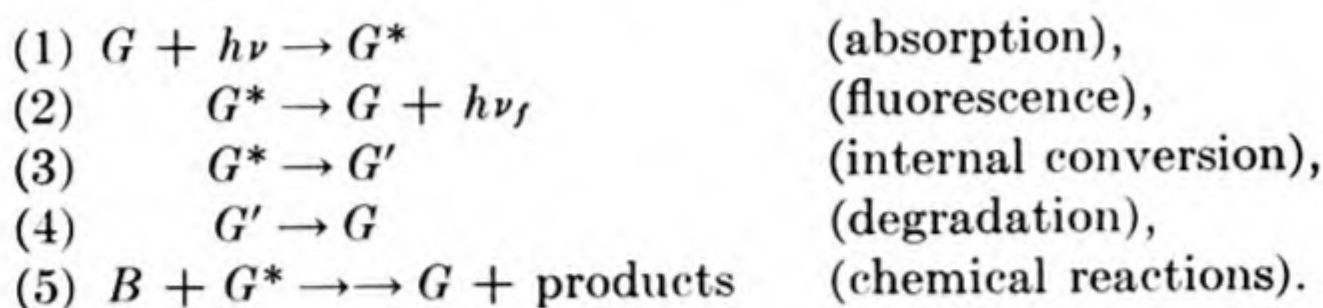
### LONG-LIVED ENERGETIC STATES

It was formerly maintained by many photochemists that fluorescence and photochemical action are strictly complementary actions. However, a large amount of information, chiefly qualitative but in part quantitative, which is definitely incompatible with this belief, has accumulated. Efficient photochemical reactions are known which involve compounds whose fluorescence yield is small, even in dilute solutions in inert solvents. Particularly in some dye-sensitized photooxidations involving weakly fluorescent sensitizers (Shpol'skiĭ and Sheremet'ev, 1936; Franck and Livingston, 1941), the absorbed photon has too small an energy to dissociate the absorbing molecule. At least in these cases a long-lived excited state must be an intermediate in the photochemical reaction.

To demonstrate this, let us consider a particular example: the auto-



oxidation of allylthiourea photosensitized by chlorophyll (Gaffron, 1927). It seems safe to assume that the reaction involves an interaction between an energy-rich chlorophyll molecule and either an oxygen or an allylthiourea molecule. If normal chlorophyll is represented by  $G$ , its fluorescent state by  $G^*$ , some long-lived excited state by  $G'$ , and the reacting molecule, either oxygen or the reducing agent, by  $B$ , a generalized (and simplified) mechanism for the reaction may be written as follows:



Expressed in appropriate units (einsteins per liter per second), the rate of step (1) is  $v_1 = I_{abs}$ . The rates of the four subsequent steps are, respectively,  $v_2 = k_2[A^*]$ ,  $v_3 = k_3[A^*]$ ,  $v_4 = k_4[A']$ , and  $v_5 = k_5[B][G^*]$ . As long as the measurements of the fluorescence and of the chemical reaction are made under the same conditions, it is immaterial whether  $k_3$  is a function of the nature of the solvent, i.e., whether the solvent quenches the fluorescence. Under steady-state conditions,

$$\frac{d(A^*)}{dt} \approx 0 \quad \text{and} \quad \frac{d(A')}{dt} \approx 0,$$

and thus

$$v_1 = v_2 + v_3 + v_5, \quad \text{and} \quad v_3 = v_4$$

may be written. Substituting for the several  $v_i$ , the following values for the fluorescence yield  $\varphi_{fl}$  and for the photochemical yield are obtained:

$$\begin{aligned}
 \varphi_{fl} &= \frac{I_{fl}}{I_{abs}} = \frac{k_2}{k_2 + k_3 + k_5[B]}; \\
 \varphi &= \frac{v_4}{I_{abs}} = \frac{k_4[B]}{k_2 + k_3 + k_5[B]}.
 \end{aligned}$$

In a dilute solution and in the absence of quenchers the fluorescence yield  $\varphi_{fl}$  for chlorophyll (Prins, 1934) is approximately 0.1. It is not detectably quenched by allylthiourea, and it requires about  $2 \times 10^{-2}$  mole of oxygen to reduce the fluorescence to half its maximum value. A quantum yield  $\varphi$  of practically unity was obtained in a solution containing about 0.1 mole of allylthiourea and 0.002 mole of oxygen. The natural mean life of the excited state must be of the order of  $10^{-7}$  sec; therefore  $k_2 \approx 10^7 \text{ sec}^{-1}$ . Since in the absence of quenchers the fluorescence yield is approximately 0.1,

$$\varphi_{fl}^0 = 0.1 = \frac{k_3}{k_2 + k_3}.$$

Therefore  $k_3$  is about  $10^8 \text{ sec}^{-1}$ . Approximately,

$$\varphi_{fl} = \frac{10^7}{10^8 + k_5[B]}$$

The addition of 0.1 mole of allylthiourea to a chlorophyll solution reduces its fluorescence by less than 5 per cent. Accordingly, if a direct reaction of this substance with the (singlet) excited chlorophyll is responsible for the sensitized reaction, then  $k_4[B] \lesssim 0.05 \times 10^8$ , or  $k_5 \lesssim 5 \times 10^7 \text{ liters mole}^{-1} \text{ sec}^{-1}$ . However, the quantum yield for the reaction is

$$\varphi = \frac{k_4[B]}{10^8 + k_5[B]}$$

In the presence of 0.1 mole of allylthiourea the quantum yield is certainly greater than 0.9, which requires that  $k_5$  be greater than  $10^{10}$ . Since the difference between these two estimates is 200-fold or more, the postulate that the reducing agent reacts directly with the singlet excited state must be rejected. If it is assumed that a collision between an oxygen molecule and an excited chlorophyll molecule is responsible for both the reaction and the fluorescence quenching, the computed values of  $k_5$  are  $10^{10}$  and  $5 \times 10^{11} \text{ sec}^{-1} (\text{moles/liter})^{-1}$ , respectively. Not only is this fiftyfold discrepancy too great to be explained in terms of experimental uncertainties, but also the value ( $5 \times 10^{11}$ ) required by the photochemical data is unreasonably large. The frequency factor for a bimolecular reaction between molecules of ordinary dimensions and mass at room temperature is given by the simple collision theory (Moelwyn-Hughes, 1947) as about  $10^{11} \text{ sec}^{-1} (\text{liter/mole})$ . Although the size of the chlorophyll molecule might possibly increase this number fivefold,  $5 \times 10^{11}$  is surely an upper limit. In order for the rate constant to be as large as the frequency factor, the steric factor must equal unity and the energy of activation must be zero. Moreover, the rate of such a reaction, occurring in solution, will be determined by the frequency with which the reaction partners can diffuse together (Fowler and Slater, 1938) and not by the total number of collisions between them. In a condensed phase, collisions occur in bursts of (probably) 100 or 1000, and the number of encounters is correspondingly reduced. It must be concluded that this reaction cannot be induced by a collision of the directly excited chlorophyll molecule with either of the reaction partners. Therefore the sensitizer molecule must be capable of existing in some long-lived energy-rich form. The same conclusion may be reached from the results of analysis of kinetic data for other sensitized reactions (Franck and Livingston, 1941).

More direct evidence is offered by the experimental investigation (Gaffron, 1937) of the photochemical autooxidation of the aromatic hydrocarbon, rubrene. Since the quantum yield for this reaction remains high even when the oxygen concentration is relatively low, it can be shown



that the half life of the excited rubrene molecule is very long (possibly 1 sec or more). This is very much greater than the maximum half life of the directly excited, singlet state. In this case, as in those sensitized reactions previously referred to, a long-lived excited state must be an intermediate in the photochemical reaction.

At the present time, there appear to be no data available from which it can be concluded that the long-lived state required by photochemical evidence is identical with the phosphorescent state, usually studied at low temperatures and in rigid media. However, in the absence of information to the contrary, this identification is commonly made as a simplifying hypothesis. In one case, that of fluorescein in boric acid glass, direct measurements of the paramagnetism of the excited substance (Lewis *et al.*, 1949) have demonstrated that the phosphorescent state is a triplet one. Comparison (McClure, 1949) of the measured half lives of the long-lived fluorescence of a large number of aromatic compounds and of a few aliphatic ketones makes it appear probable that the phosphorescent states of these compounds are likewise triplet states.

It should not be assumed that all photochemical reactions involve either dissociation or a long-lived excited state. Benzene and some of its methyl derivatives react photochemically with molecular oxygen to form peroxides. Their limiting fluorescence yields are high, and their fluorescence and photochemical reactions are complementary functions of oxygen concentration (Bowen and Williams, 1939). It is probable, therefore, that the photochemical reaction in these cases goes by way of a direct interaction between an oxygen molecule and the singlet excited, i.e., fluorescence, level of the hydrocarbon molecule (Kasha and Nauman, 1949).

### QUENCHING OF EXCITED STATES

A low quantum yield for a photochemical reaction may, under some conditions, be the result of the quenching of the excited molecule by impurities, reaction products, the solvent, or even one of the reactants. Such quenching brings about a decrease in the fluorescence yield, which is not accompanied by an observable chemical reaction. The mechanism of the process has been the subject of much speculation (Pringsheim, 1949, p. 335), and it appears probable that no one explanation is consistent with all the experimental facts. In some cases the quenching appears to be the result of the reversible formation of nonfluorescent complexes, whereas in others it may be due to collision (or the close approach) of a quencher and excited molecule. It is possible (Rollefson and Stoughton, 1941; Livingston and Ke, 1950) that the quencher acts by inducing a transition of the potentially fluorescent molecule from its excited singlet state to its lowest triplet state. Fluorescence quenching of this type would not necessarily be accompanied by a corresponding reduction in the quantum yield of a photochemical reaction.

At moderately high concentrations ( $10^{-3} M$  or greater), the fluorescence of most compounds decreases as the concentration is increased. In some cases this self-quenching is due to the reversible formation of nonfluorescent dimers (Rabinowitch and Epstein, 1941; Lewschin, 1935). This is by no means invariably true. More generally, self-quenching appears to be related to the "migration of excitation energy" (Vavilov, 1943; Förster, 1948, 1950; Franck and Livingston, 1949). As a consequence of this effect, photochemical reactions which do not involve direct optical dissociation or predissociation should be expected to become correspondingly inefficient at high concentrations of the light absorber (cf. Gaffron, 1927).

### TRANSFER OF EXCITATION

Transfer of energy of excitation between like or unlike molecules may well play an important role in photochemistry, particularly in heterogeneous systems and in solutions in which the concentration of the nonabsorbing reactant is relatively small. In crystals such a transfer of excitation may be caused by migration of electrons in a conductivity band of the crystal (Franck, 1948), by "exciton migration" (Frenkel, 1931), or by a radiationless transfer which may be called "classical resonance" (Franck and Livingston, 1949). In a solution such a transfer of excitation can occur only on collision or as the result of classical resonance. This latter possibility has been carefully analyzed by Förster (1948), who has shown that for certain dyes and pigments such a transfer can occur efficiently at distances as great as 50 or 100 Å. Sensitized fluorescence, in which the photon is absorbed by one molecule and the energy transmitted to an unlike molecule which emits its characteristic fluorescence, is one consequence of classical resonance. It is a well-established phenomenon in gases at low pressure, such as mixtures of mercury and thallium (Cario and Franck, 1923), and has been reported for at least one case in liquid solutions (Watson and Livingston, 1950). Transfer of excitation between like molecules can be most readily detected by the depolarization (Vavilov, 1943; Förster, 1948) of fluorescence which is excited with plane polarized light. In some cases, self-quenching (Watson and Livingston, 1950) and quenching by added substances (Förster, 1950) appear to be the consequence of classical resonance.

Energy of excitation may be exchanged between different groups within a complex molecule by a similar mechanism (Franck and Livingston, 1949). Weissmann (1942) made the interesting observation that light which is absorbed by the organic part of the europium salicylaldehyde molecule excites fluorescence characteristic of the europium ion; this effect is very probably the result of such a radiationless transition between the separate parts of the molecule. When the carbon monoxide-myoglobin complex is illuminated with light of either 5400 or 2800 Å, carbon monoxide is split off, and the quantum yield of the process is about unity



(Bücher and Kaspers, 1947). Myoglobin is an enzyme, consisting of two hemin groups attached to a protein having a molecular weight of about 32,000. The absorption at 5460 Å is due almost entirely to the hemin group, and the corresponding photochemical dissociation is presumably either predissociation or direct optical dissociation. At 2800 Å, much of the absorption (about 40 per cent) is due to the tyrosine and tryptophane groups which are presumably distributed throughout the protein molecule. It is very probable that the energy is transferred from these primarily excited groups to the hemin-carbon monoxide complex by classical resonance. The effect is almost certainly not caused by a general degradation of the protein, since the quantum efficiencies of such reactions are smaller by several orders of magnitude (Finkelstein and McLaren, 1949).

### SOLVENT EFFECTS

The presence of a solvent may affect the primary process in several different ways; namely, it may decrease, leave unaltered, or even increase the quantum yield of the photochemical reaction. The solvent may also bring about a change in the reaction products. What the effect will be in a specific instance cannot, in general, be predicted. However, a knowledge of the possible influences of a solvent on the primary step is of great value in interpreting experimental results.

In one sense the simplest, and perhaps the most important, effect of the solvent on the primary process is its influence on the normal equilibrium state of the reactant (i.e., absorbent) molecules. Acidic or basic media often determine the charge on solute molecules and thus alter their absorption spectra and their relative probabilities of fluorescing, dissociating, undergoing internal changes, or degrading their energy of excitation. Changes in the molecular state, such as dimerization or the formation of molecular complexes with the solvent, are of even more common occurrence and are likewise effective in changing the photochemical properties of the solute. Usually such changes can be detected by measurements of nonkinetic properties, such as absorption spectra, conductivity, or transference, and the colligative properties of the solution.

Since solute molecules are continuously in a state of multiple impact with the surrounding molecules, any oscillational energy which the solute molecule may possess, in excess of the thermal equilibrium amount, is quickly drained off by successive collisions of the second kind. This loss of oscillational energy may stabilize an electronically excited molecule by reducing its probability of predissociating or of undergoing internal conversion. Conversely, the solvent may "induce predissociation" of a molecule by light of wave lengths too long to bring about dissociation of the isolated molecule (Herzberg, 1950). After an act of internal conversion, a complex molecule has momentarily a large fraction of its energy of excitation in its generalized oscillational degrees of freedom. If all its

excitation energy is present as oscillational energy (i.e., if the energy-rich molecule is in its electronic ground state), the solvent will increase the probability of this energy being lost to the surroundings as heat and so reduce the quantum yield. Many dyes and other complex molecules go (by internal conversion) from their excited singlet states to a relatively long-lived (triplet) energetic state, which is chemically an activated state. Under these latter conditions, deactivation by collisions of the second kind has little, if any, effect on the probability of reaction.

### CAGE EFFECT

When a dissolved molecule dissociates, its fragments (atoms or radicals) are surrounded by a barrier of solvent molecules. Held in this cage, they will make a number of collisions with one another before they can move out of the cage. This increases the probability that they will recombine and so reduces the efficiency of the photochemical reaction. There are several factors which influence the importance of this "cage effect." When the absorbed photon has greater energy than is required to dissociate the molecule, the excess kinetic energy of the resulting atoms will increase their chance of escaping from the cage. This chance of escape is greater for small atoms (especially hydrogen atoms) than it is for larger radicals. In the case of large radicals, there is a compensating factor. The recombination of large radicals requires some (small) energy of activation and demands that very strict conditions of relative orientation be fulfilled. Both these requirements greatly reduce the probability of recombination at a collision and correspondingly decrease the importance of the cage effect. Experimental investigation (Rollefson and Burton, 1939, Chap. XIV) of the cage effect demonstrates that it is real and can be of importance in photochemical reactions. However, it appears to be unexpectedly specific.

A solvent may change the nature of the reaction products either by reacting (in one or more secondary steps) with the primary products or by altering the relative probabilities of alternative primary reactions. An excited complex molecule can dissociate either into two radicals or into two stable molecules. It has been reasonably substantiated (Bamford and Norrish, 1938) that certain ketones undergo both types of dissociation to a comparable extent in the gas phase but, in solution, dissociate only into stable molecules. This difference in products has been attributed to the action of the solvent, which cages in the free radicals and so brings about their recombination.

### PHOTOCHEMICAL TRANSFER OF ELECTRONS OR PROTONS TO THE SOLVENT

In condensed systems the absorption of a photon of ultraviolet or even visible light can lead directly to the formation of an ion by the ejection of



either an electron or a proton. An important special case of this type is the photoconductivity of ionic crystals (Mott and Gurney, 1940). Although this latter phenomenon is of vital importance in determining the photochemical and optical properties of crystal phosphors, it appears to be too specialized for this discussion.

When certain compounds dissolved in glassy media at low temperatures are illuminated with ultraviolet light, semiquinones are formed by the ejection of an electron from the absorbing molecule (Lewis and Bigeleisen, 1943). In these low-temperature rigid solutions the return of the electron is greatly retarded, and the spectrum of the resulting semiquinone can be directly measured. In some instances the semiquinone is stabilized by the thermal loss of a proton to the surroundings. A number of organic compounds (all of which contain basic nitrogen, sulfur, or oxygen) exhibit this property. There can be no reasonable doubt that a similar photoionization can occur in aqueous solutions. The near-ultraviolet absorption of ferrous ion is very probably due to the transfer of an electron from the central ion to the surrounding shell of water molecules (Zimmerman, 1949). Unless this excited system is stabilized by a secondary reaction, such as the elimination of elementary hydrogen or the reduction of an oxidizing agent, it will return after a short time to its original state.

The transfer of a proton from an absorbent molecule to a solvent molecule or between the two components of a molecular complex has been demonstrated by three independent methods. If aqueous solutions of organic acids or bases are exposed to ultraviolet radiation which does not decompose them, it has been reported (Terent'ev, 1949) that the pH of the solution changes reversibly. In the majority of the cases studied, the pH increased upon illumination by a few tenths of a unit. This suggests that the excited molecules are weaker acids than the corresponding normal molecules.

More detailed and systematic measurements were made by Förster (1950) on solutions of hydroxy and amino derivatives of sulfonated pyrenes. These compounds display either one or the other of two different absorption spectra and of two different fluorescence spectra, depending on the pH of the solution. The shifts in the emission and absorption spectra do not occur in the same pH range. From an analysis of these spectral shifts the ionization constants of the normal and excited molecules can be determined. The changes in the ionization constants, due to electronic excitation, are surprisingly great. For example, the ionization constant of hydroxy trisulfonated pyrene is increased by a factor of  $10^7$  upon excitation. Since the acid-base equilibrium is realized during the lifetime of the excited (fluorescent) state, this photochemically induced ionization must be classed as a primary step. It does not involve internal conversion.

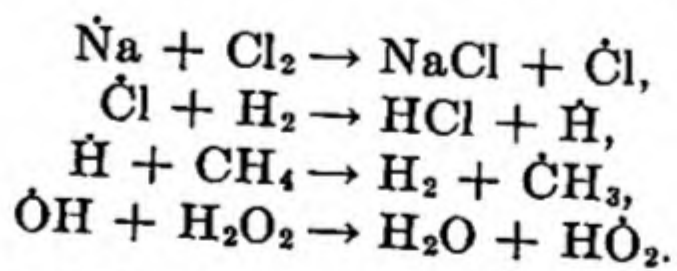
Terenin and Kariakin (1947) prepared thin films by subliming simultaneously acridine and an organic acid (e.g., succinic acid) onto a plate kept at  $-180^{\circ}\text{C}$ . When irradiated with wave length 3660 Å, the newly prepared films exhibited the violet fluorescence which is characteristic of neutral acridine. When the material had stood for some time at  $-180^{\circ}\text{C}$ , or for a much shorter time at room temperature, this fluorescence was replaced by the green emission which is given by acridinium salts. A short irradiation with wave length 2537 Å restored the film to its violet fluorescent state. On standing in the dark the fluorescence again became green. These changes could be repeated indefinitely. The effect of irradiation with actinic light must be the transfer of a proton across the hydrogen bond from the amino group to the carboxyl ion. This reaction is essentially similar to those which were studied in aqueous solution. They all involve the transfer of photons across hydrogen bonds. Terenin (1947) has suggested that processes of this type may play an important role in the photochemistry of biological systems.

### SECONDARY STEPS

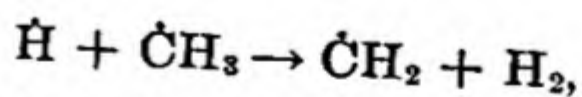
The photochemical secondary steps, those simple reactions which transform the primary products into the stoichiometric reaction products, are identical with the reaction steps which are responsible for the observed kinetics of thermal (dark) reactions (Laidler, 1950, Chap. 7). The individual steps are kinetically simple—commonly unimolecular, bimolecular, or termolecular chemical reactions. Under some conditions the kinetics of the over-all reaction are influenced by the rates of the diffusion, adsorption, or desorption processes.

### BIMOLECULAR STEPS

Bimolecular reactions involving a reactive entity and a normal molecule are of special importance in the mechanisms of photochemical reactions. Reactions between stable molecules and atoms have been extensively investigated. The following reactions are typical examples whose existence has been reasonably well established (Steacie, 1946; Polanyi, 1932; Laidler, 1950):



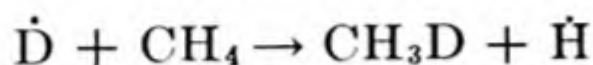
Similar reaction steps such as



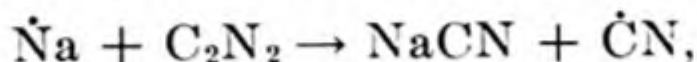
which involve a radical in place of the stable molecule, appear to occur with equal facility. Reactions of this general type, which do not change



the total number of particles involved, are truly bimolecular and can occur in dilute gas as readily as in a condensed system. When such reactions are exothermic, their heats of activation are usually low, ranging from 0 to 10 kcal. It does not follow that any such reaction which can be postulated will occur in practice. For example, the reaction

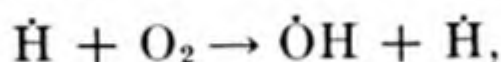


apparently does not take place under ordinary conditions, and theoretical calculations indicate that its heat of activation may be as high as 40 kcal. The reaction between atomic sodium and cyanogen,



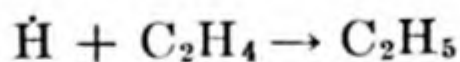
is slow owing to the smallness of its probability factor, although its energy of activation is approximately zero.

When sufficient energy is available an atom may react with a molecule to form two radicals. A step of this kind (Lewis and von Elbe, 1938),

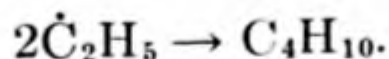


undoubtedly plays an important role in the explosive combination of hydrogen and oxygen.

Combination of two atoms results in the formation of a molecule possessing more oscillational energy than is necessary to dissociate it. As a result, the life of the quasi molecule is equal to the time of a single oscillation (about  $10^{-13}$  sec), and at ordinary pressures it dissociates before it has a chance to make a stabilizing collision with a normal molecule. In other words, combination of atoms in the gas phase as a bimolecular reaction cannot occur. However, two radicals, or an atom and a moderately large radical, can combine to form a molecule whose energy can be distributed among several degrees of freedom and whose mean life will therefore be comparable to the time between collisions in an ordinary gas. Examples of this type are

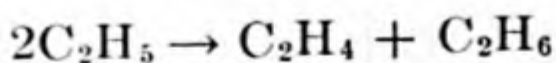


and



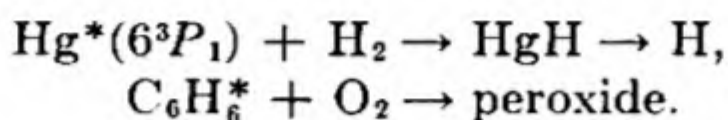
There is, of course, no restriction on the combination of atoms in a condensed system, since the colliding atoms will be continuously in collision with the surrounding solvent molecules.

Disproportionation and probably metathetical reactions occur and influence the mechanisms of reactions. The reaction



is detectable under suitable conditions but is apparently less probable than the simple combination of the radicals to form butane.

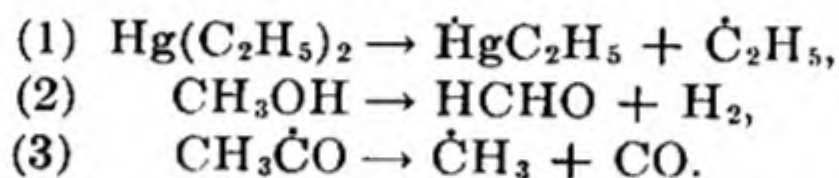
Secondary steps are, of course, not restricted to reactions involving radicals; the reactive species may be a relatively unstable molecule or an excited atom or molecule. In some cases the excited molecule in a singlet, fluorescent state may enter into the reaction, as is illustrated by



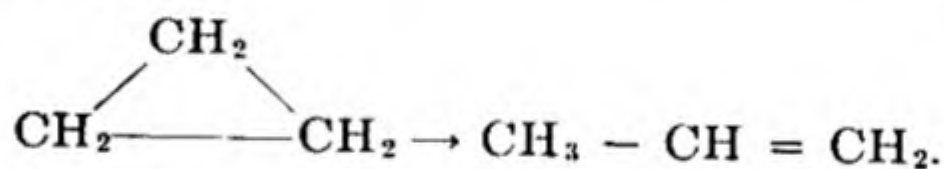
Most photochemical reactions of complex molecules, such as the autooxidation of rubrene, which take place by way of an excited state appear to involve a long-lived (possibly triplet) excited state.

### UNIMOLECULAR STEPS

True unimolecular reaction steps are limited to complex radicals or molecules. They can result in the formation of (1) two radicals, (2) two stable molecules, or (3) a stable molecule and a radical:



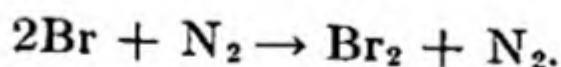
Internal rearrangements of complex molecules can be the result of unimolecular reactions. Examples of this type, which have been studied, include *cis-trans* isomerizations, racemizations (probably), as well as reactions of the following type:



Many first-order reactions which occur in solution involve a molecule of the solvent and are therefore bimolecular rather than unimolecular reactions.

### TERMOLECULAR STEPS

With few possible exceptions, termolecular gas-phase reactions are recombinations of atoms (or radicals), occurring as three-body collisions involving some other molecule or radical, i.e.,



In solution, termolecular reactions involving one or more molecules of the solvent are probably of much more frequent occurrence.

### DIFFUSION-CONTROLLED PROCESSES

The rates of some reaction steps which take place in condensed systems or in gases at moderate or high pressures are controlled by diffusion processes. Most of these reactions involve heterogeneous or microheterogeneous media. There is an important class of such reactions which occur



in homogeneous, condensed phases. Steps of this type have very low heats of activation and steric factors of the order of magnitude of unity; accordingly, practically every collision results in reaction. Examples of this type are the combination of atoms or small radicals, the quenching of the fluorescence of dyes by efficient quenchers, and enzymatic reactions at low concentrations of the substrate. These reactions occur at every encounter of the reactant molecules. The frequency of encounters, unlike that of collisions, is determined by the rate of diffusion, which in turn is dependent on the viscosity of the solution.

Under the normal conditions in which gas-phase reactions are commonly studied, recombination of atoms frequently is a wall-catalyzed process, whose rate is fixed by the rate of diffusion of the atoms to the wall of the vessel. The rate of atomic recombination  $2A \rightarrow A_2$ , occurring both by three-body collisions and by wall catalysis, can be represented by an equation of the following form (Kassel, 1932, pp. 176-180):

$$\frac{d[A_2]}{dt} = kP_c[\dot{A}]^2 + \frac{k'}{P_D} [\dot{A}].$$

The factors  $P_c$  and  $P_D$  are linear functions of the partial pressures of the components of the gas. The coefficient  $k'$  is influenced by the geometry of the vessel, becoming greater as the surface-to-volume ratio of the vessel increases. In a photochemical steady state the homogeneous recombination (whose rate is proportional to the square of the concentration of atoms) is favored by an increase in the intensity of the absorbed light.

If atoms or radicals are formed in a liquid-phase photochemical reaction, the observed quantum yield is likely to be influenced by the rate of stirring of the solution since, in an unstirred solution, the steady-state concentration of atoms will, in general, be spatially nonuniform. This effect is especially important if a large fraction of the actinic light is absorbed in a thin film near the window through which the light enters.

## MECHANISM OF COMPLEX REACTION

### GENERAL PROBLEM

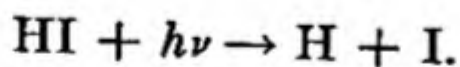
Few, if any, chemical reactions are kinetically simple in the sense that they involve only one reaction step which is of simple order and which is identical with the stoichiometric reaction. The observable course of a photochemical reaction is the result of the simultaneous occurrence of a number of reaction steps. A set of reaction steps, which is consistent with the stoichiometry and kinetics of a reaction, constitutes the mechanism of the reaction. The kinetic equation for the over-all reaction can be obtained by combining the rate equations for the several steps in such a way that the concentrations of the reaction intermediates are eliminated.

For relatively simple reactions (Kassel, 1932; Laidler, 1950) this can be done by solving simultaneously the differential rate equations for the individual steps. In general, the resulting solution cannot be obtained in terms of simple functions, and the analysis demands a mathematical skill which the average photochemist does not possess. As a result it has become conventional among students of kinetics to use approximate methods to deduce the over-all rate equation from a postulated mechanism.

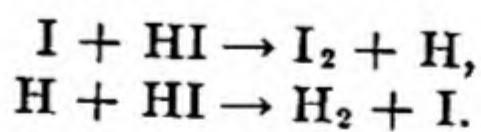
### STEADY-STATE APPROXIMATION

The most generally applicable of these simple methods is the so-called "steady-state approximation." A steady state may be defined as a condition in which the rates of change of the concentrations of the several intermediates are very small compared to the rates of change of the concentrations of the reactants and products. This condition is realizable whenever the ratio of the concentrations of the intermediates to the concentrations of the reactants is very much less than unity. When this condition is not attained, the method is not applicable; however, it should not then be necessary since the (larger) concentrations of the intermediates could be measured by experimental means. In no reaction is the steady state attained instantaneously. However, the time required for its attainment is usually a negligibly small fraction of the half time of the reaction. The steady-state approximation consists in setting the rates of change of each of the intermediates equal to zero and in solving simultaneously the resulting algebraic equations. This process can be explained most easily by outlining the details of two well-known examples.

*Examples.* The decomposition of hydrogen iodide is a classic example (Warburg, 1916; Bonhoeffer and Farkas, 1928) of a carefully studied and thoroughly understood photochemical reaction. Although this reaction is so simple that it is not necessary to use the steady-state approximation method in its analysis, it will serve to introduce the fundamentals of this procedure. Gaseous hydrogen iodide strongly absorbs light of wave length 3000 Å or shorter. It is nonfluorescent, and its absorption spectrum is continuous, showing no vibrational or rotational structure. It follows from these observations that the primary act is one of direct optical dissociation,



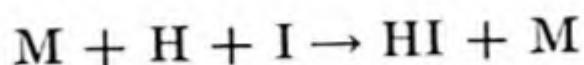
The secondary steps must be reactions of hydrogen and of iodine atoms. They may combine to form hydrogen iodide or molecular hydrogen and iodine, or more probably they may react with hydrogen iodide:



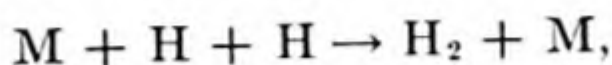
The first of these two reactions can be ruled out since it is strongly endo-



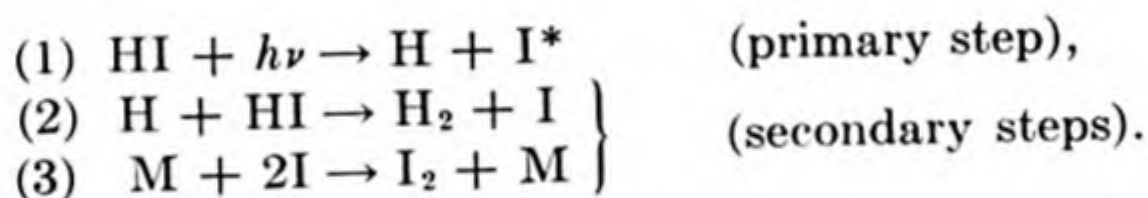
thermic ( $\Delta H = 34$  kcal). The second, being an exothermic reaction between an atom and a diatomic molecule, should have a small heat of activation and a steric factor not differing greatly from unity. The two possible alternative reactions that a hydrogen atom can undergo,



and



can occur only by three-body collisions with any molecule,  $M$ , or by diffusion to the wall. The relative importance of these atomic combinations is further reduced by the fact that the steady-state concentrations of the atoms must be much smaller than the concentration of the reactant, hydrogen iodide. Since the hydrogen atom concentration is kept very small by its efficient reaction with hydrogen iodide, the only reaction which iodine atoms can enter into, appreciably, is association to form molecular iodine. Accordingly, the mechanism for the reaction may be written as follows:



Expressing the "intensity"  $I_{abs}$  of the absorbed light in the photochemical units of einsteins per liter per second and the rates of the chemical reactions in moles per liter per second, the rate equations for the three steps of the mechanism may be written

$$\begin{aligned} v_1 &= I_{abs}, \\ v_2 &= k_2[HI][H], \\ v_3 &= k_3P_c[I]^2. \end{aligned}$$

The rate of decomposition of hydrogen iodide is the sum of the rates of steps (1) and (2),

$$-\frac{d[HI]}{dt} = v_1 + v_2 = I_{abs} + k_2[HI][H].$$

Introducing the steady-state assumption

$$\frac{d[H]}{dt} = 0,$$

then

$$I_{abs} = k_2[HI][H]$$

may be written. Therefore

$$-\frac{d[HI]}{dt} = 2I_{abs}.$$

The quantum yield  $\varphi$  expressed in terms of molecules of hydrogen iodide decomposed is

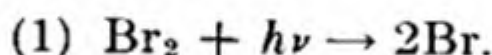
$$\varphi = - \frac{d[\text{HI}]/dt}{I_{\text{abr}}} = 2.$$

This is in excellent agreement with the experimentally determined quantum yield of 2.00, the value which has been observed over a wide range of conditions, including pressures down to 0.008 mm Hg. There can be no reasonable doubt of the correctness of this mechanism.

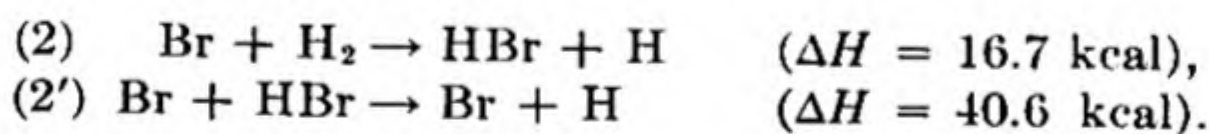
The thermal formation of hydrogen bromide was perhaps the first reaction to which the steady-state approximation method was applied (Christiansen, 1919; Herzfeld, 1919; Polanyi, 1920). At temperatures in the range from 150° to 200°C, a photochemical formation of hydrogen bromide can be observed which is relatively free from disturbance by the thermal reaction. The quantum yield of this reaction is the following function of concentrations and intensity (Bodenstein and Lütkemeyer, 1924):

$$\varphi = \frac{d[\text{HBr}]/dt}{I_{\text{abr}}} = \frac{k_{\varphi}[\text{H}_2]}{I_{\text{abs}}^{\frac{1}{2}} \left\{ 1 + \frac{[\text{HBr}]}{k'[\text{Br}_2]} \right\}}.$$

Absorption of blue or near-ultraviolet light leads to optical dissociation of bromine. The primary step of this reaction is therefore



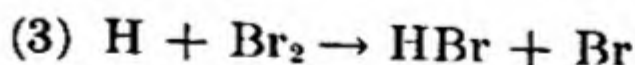
The bromine atoms might be expected to react with either molecular hydrogen or hydrogen bromide:



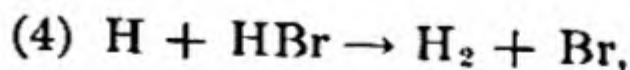
Both these reactions are endothermic, with the heats of reaction indicated. Since the steric factors for these two reactions are very probably of the same order of magnitude, the rate of the second reaction should be smaller than that of the first by a factor of about

$$\frac{e^{-40,600/2T}}{e^{-16,200/2T}} = e^{-24,400/2T}.$$

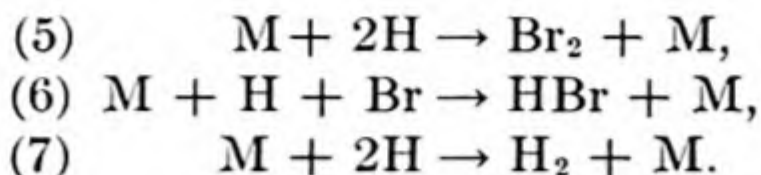
In the temperature range under consideration this is a very small number, and the second reaction may be justifiably dropped from consideration. The hydrogen atoms, formed in step (2), can undergo similar reactions,



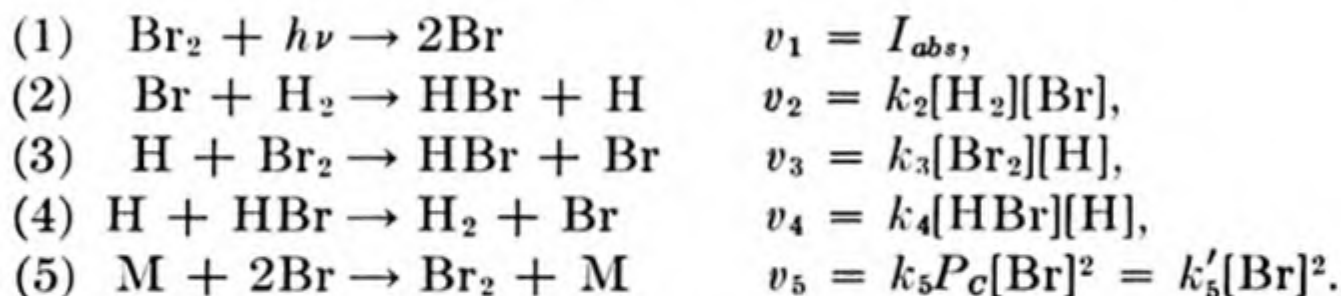
and



both of which are exothermic. None of these reactions reduce the total number of atoms present. Therefore the formation of atoms in step (1) must be balanced by their disappearance in atomic combination reactions:



Since no hydrogen atoms are formed directly and since, furthermore, steps (3) and (4) are much more efficient than the endothermic step (2), the concentration of hydrogen atoms must be much less than that of bromine atoms. Accordingly, the rates of step (6) and (7) must be small compared to the rate of step (5). As a working hypothesis, let us assume that only steps (1) through (5) affect the course of the reaction. The corresponding mechanism may be written as follows, where the expression for each rate follows its chemical equation:



Introducing the steady-state assumptions

$$\frac{d[\text{Br}]}{dt} = 0 \quad \text{and} \quad \frac{d[\text{H}]}{dt} = 0,$$

then

$$2v_1 + v_3 + v_4 = v_2 + 2v_5$$

and

$$v_2 = v_3 + v_4$$

may be written. Therefore

$$v_1 = v_5$$

or

$$[\text{Br}] = \left( \frac{I_{abs}}{k'_5} \right)^{1/2}.$$

Similarly,

$$k_2[\text{H}_2][\text{Br}] = \{k_3[\text{Br}_2] + k_4[\text{HBr}]\}[\text{H}]$$

and

$$(\text{H}) = \frac{k_2[\text{H}_2]}{k_3[\text{Br}_2] + k_4[\text{HBr}]} \left( \frac{I_{abs}}{k'_5} \right)^{1/2}.$$

In terms of the mechanism the rate of formation of hydrogen bromide is given by

$$\begin{aligned}\frac{d[\text{HBr}]}{dt} &= v_2 + v_3 - v_4 \\ &= k_2[\text{H}_2][\text{Br}] + \{k_3[\text{Br}_2] - k_4[\text{HBr}]\}[\text{H}],\end{aligned}$$



Introducing the values for the concentrations of the intermediates and simplifying,

$$\frac{d[\text{HBr}]}{dt} = \frac{\frac{2k_2}{(k'_5)^{1/2}} I_{abs}^{1/2} [\text{H}_2]}{1 + \frac{k_4[\text{HBr}]}{k_3[\text{Br}_2]}}$$

The corresponding equation for the quantum yield,

$$\varphi = \frac{d[\text{HBr}]/dt}{I_{abs}} = \frac{\frac{2k_2}{k'_5} [\text{H}_2]}{I_{abs}^{1/2} \left\{ 1 + \frac{k_4[\text{HBr}]}{k_3[\text{Br}_2]} \right\}}$$

is identical in form with the empirical equation. The empirical constant  $k'$  corresponding to  $k_3/k_4$  of the theoretical equation is independent of temperature over a wide range. This is consistent with the mechanism since steps (3) and (4) are exothermic reactions of an atom with a diatomic molecule and should have small heats of activation. The observed heat of activation for  $k_\varphi$  is 17.6 kcal. This is only slightly greater than the (endothermic) thermochemical heat of step (2). The mechanism has been further tested by investigating the effect of such substances as inert gases on the photochemical rate and by comparing the photochemical to the thermal rate. The results of all these tests are consistent with the proposed mechanism. It may be concluded, therefore, that it is very probably correct.

*Limitations of the Method.* In the two examples just considered the determination of the mechanism was simplified by rejecting possible reaction steps on the basis of thermochemical data. Frequently the necessary thermal data are not available, and there is no a priori reason for rejecting any of the chemically possible steps. Under these conditions the kineticist endeavors to find a mechanism consisting of a minimum number of reaction steps which is consistent with the stoichiometry and the kinetics of the reaction. Whenever possible, the rate constants for the individual steps are evaluated in terms of the empirical rate equation. Unless these several constants fall within the (frequently rather wide) range of values permitted by rate theory, the mechanism must be rejected. If the mechanisms of different reactions have steps in common, the rate constants for these steps should have the same values regardless of what mechanism they occur in.

There is no general method by which the mechanism of a reaction may be derived from empirical rate data. The process of devising a mechanism is essentially a "cut-and-try" procedure, in some respects similar to the methods used in solving differential equations. As in the latter case,

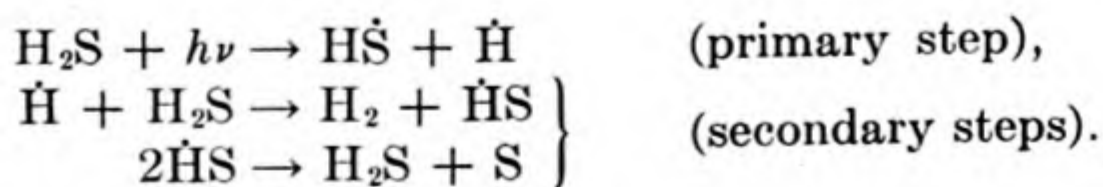
certain general rules and analogies are helpful in guiding the intuition of the kineticist. It is rarely true that a reaction mechanism can be regarded as true or even as very probably true. Usually all that can be claimed for a mechanism is that it is consistent with all pertinent information. This does not preclude the possibility that some other mechanism, or even many other mechanisms, may likewise be compatible with the data. A number of reaction mechanisms which were at one time tentatively accepted had to be discarded later when more information became available. In spite of these unsatisfactory characteristics of the mechanisms of complex reactions, there appears to be no way of obtaining information about complex reactions other than by postulating and testing mechanisms.

## EXAMPLES OF THE PRINCIPAL TYPES OF PHOTOCHEMICAL REACTIONS

The types of photochemical reactions which have been studied most extensively are decompositions, oxidations, polymerizations, hydrolyses, and internal rearrangements. A great part of the early photochemical literature is devoted to oxidations, especially chlorinations. Much of the classical information about chain reactions was derived from these latter measurements. From a biological point of view the details of these investigations are of little immediate interest, and for this reason they are omitted from the following discussion.

### DECOMPOSITION REACTIONS

The photochemical decomposition of a wide variety of compounds, ranging in complexity from hydrogen iodide to complex azo dyes, has been investigated, in many cases with considerable care. The decomposition of hydrogen sulfide is a good example of a carefully studied reaction of a simple molecule. The absorption of hydrogen sulfide is continuous, becoming appreciable at about 2800 Å and reaching a maximum near 1900 Å. The corresponding primary act is presumably one of direct optical dissociation, yielding a hydrogen atom and a hydrosulfide radical. Photochemical measurements (Forbes *et al.*, 1938) made with radiation wave length of 2080 Å demonstrate that one molecule of hydrogen sulfide is decomposed for each photon absorbed, over a wide range of pressures and of light intensities. The following mechanism is consistent with all available reliable information and, for this simple system, is probably correct:



The photolysis of a variety of aldehydes and ketones has been investigated extensively and in detail (Noyes and Leighton, 1941). The results



of these studies are far from simple. It appears to be fairly definite that two different kinds of primary acts can occur. The excited molecule can dissociate either into radicals or into two such stable molecules as a hydrocarbon and carbon monoxide. The formation of the radicals is probably a process of predissociation, and the production of molecules, the result of internal conversion. Croton aldehyde, at temperatures below 150°C, is photochemically stable in spite of the facts that it is nonfluorescent and that its absorption is continuous. Although this failure to react is conceivably the result of rapid recombination of radicals formed in the primary act, it seems much more likely that the energy of excitation is lost by way of an act of internal conversion, followed by degradative collisions, i.e., collisions of the second kind, with surrounding molecules.

The photochemical decomposition of formaldehyde is probably as simple a reaction of this type as has been studied. Although the results of the several investigations of this reaction (Steacie, 1946) do not agree in all particulars, the broad outline of the mechanism appears to have been reasonably well established. The products of the reaction are carbon monoxide and molecular hydrogen. At 110°C the quantum yield is approximately unity for wave lengths from 2600 to 3500 Å. At the longer wave lengths the absorption spectrum shows fine structure but corresponds to a region of predissociation in the shorter wave-length range. The yield increases with increasing temperature, reaching a value of about 100 at 350°C. Hydrogen atoms can be detected under all experimental conditions, but there is some evidence that there is an appreciable direct formation of molecular hydrogen when the gas is illuminated with light in the longer wave-length region. The following mechanism seems to be compatible with the published results:

- |     |   |                                      |
|-----|---|--------------------------------------|
| (1) | $\text{HCHO} + h\nu \rightarrow \dot{\text{C}}\text{HO} + \dot{\text{H}}$         | (chief primary step),                |
| (2) | $\text{HCHO} + h\nu \rightarrow [\text{HCHO}] \rightarrow \text{CO} + \text{H}_2$ | (primary step at long wave lengths), |
| (3) | $\text{H}\dot{\text{C}}\text{O} \rightarrow \dot{\text{H}} + \text{CO}$           | (chain-carrying secondary step),     |
| (4) | $\dot{\text{H}} + \text{HCHO} \rightarrow \text{H}_2 + \dot{\text{C}}\text{HO}$   | (chain-carrying secondary step),     |
| (5) | $\text{M} + 2\dot{\text{H}} \rightarrow \text{H}_2 + \text{M}$                    | (chain-breaking secondary step).     |

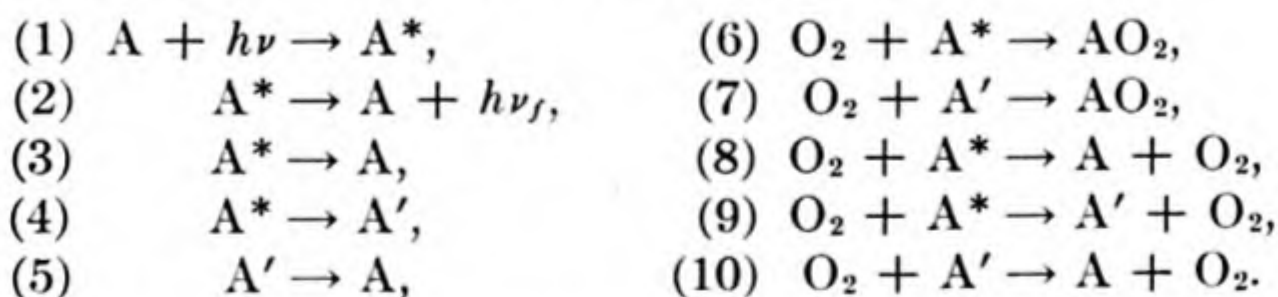
Steps (3) and (5) can occur either in the gas phase or by diffusion to the wall. For the gas-phase reaction the heat of activation of step (3) is about 13 kcal. There are, of course, a number of other possible steps, but these five are sufficient to explain the available data.

#### REACTIONS OF MOLECULAR OXYGEN

In biological systems those photooxidative reactions which involve molecular oxygen are by far the commonest. The primary step of such a

reaction may be either the optical dissociation of the oxygen molecule or the photoexcitation or dissociation of a molecule of the reducing agent. Since oxygen absorbs chiefly at wave lengths less than 1800 Å, reactions of the first type are limited to relatively transparent substrates such as hydrogen or carbon monoxide (Noyes and Leighton, 1941, pp. 246-254). In those cases where the reducing agent absorbs the light, the initially reactive species may be an excited singlet (i.e., fluorescent) state, a long-lived (triplet) excited state, or a pair of radicals, produced by some type of photodissociation.

Solutions of aryl hydrocarbons in hexane or similar solvents are fluorescent. In the presence of oxygen, their fluorescence is quenched and peroxides are formed (Bowen and Williams, 1939). With few exceptions the sum of the fluorescent yield and the peroxide quantum yield is distinctly less than unity, in some cases being as small as 0.1. None of the data for the 14 hydrocarbons investigated by Bowen and Williams are consistent with the view that the only effect of oxygen is to quench the fluorescence by reacting with the excited molecule (in its singlet, fluorescent state) to form a peroxide. Apparently oxygen can quench the fluorescence of these molecules without forming any detectable product. In five cases (benzene, *m*-xylene, fluorene, acenaphthene, and triphenylmethane) the evidence is compatible with the postulate that only the singlet, fluorescent state is involved in the peroxide formation. For the others (especially hexamethyl benzene, anthracene, naphthacene, toluene, and *p*-xylene) the experimental results strongly indicate that some or all of the peroxide is formed by a reaction between an oxygen molecule and an energy-rich nonfluorescent (triplet?) state of the hydrocarbon. This is particularly obvious for hexamethylbenzene, where a quantum yield of peroxide formation almost fivefold greater than the maximum fluorescent yield was observed. The preceding conclusions are based on the assumption that only the 10 following reaction steps occur. In these equations,  $A^*$  stands for the singlet, excited state and  $A'$  for the triplet state of the hydrocarbon molecule,  $A$ .

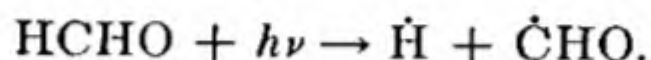


Oxidations which are initiated by the photochemical dissociation of the reductant frequently exhibit the characteristics of chain reactions. Their quantum yields are functions of the temperature, of the concentrations of reactants and products, and sometimes of the intensity of the absorbed light. Often the products are complex, and the relative amounts of the several compounds formed vary with the conditions. The detailed

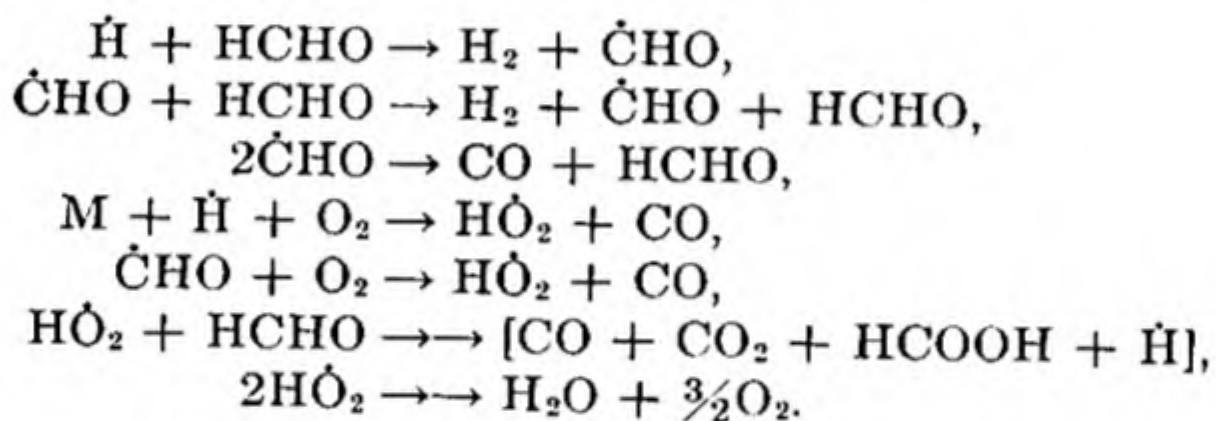


mechanism of the reaction for such a case has not been established with reasonable certainty.

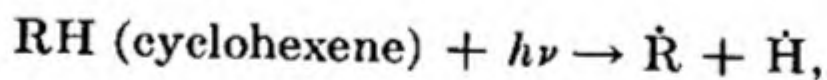
The photochemical oxidation of formaldehyde (Style and Summers, 1946) is a good example of this type of reaction. It has been studied in the temperature range of 100° to about 275°C and at a variety of pressures and compositions. Its principal products are CO, CO<sub>2</sub>, HCOOH, H<sub>2</sub>, and H<sub>2</sub>O. Their several yields vary from values of less than 1 to 30 or 40, depending in a complex way on the conditions. It is well established that  $\dot{\text{H}}$ ,  $\dot{\text{C}}\text{HO}$ , and  $\text{H}\dot{\text{O}}_2$  are reaction intermediates. The (chief) primary process is



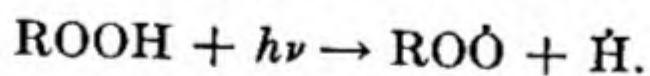
Although the detailed mechanism is not known, the experimental evidence is compatible with the postulate that the following reactions serve as (some of) the secondary steps of the reaction:



Organic peroxides are the principal products of some reactions of this general type. Peroxides may also serve as photochemical sensitizers. For example, the chief product of the photochemical oxidation of cyclohexene is the corresponding peroxide. As the concentration of the peroxide builds up in an illuminated solution containing oxygen and cyclohexene, the peroxide absorbs an increasing amount of the incident light, and the reaction is accelerated (Bateman and Gee, 1948). These observations are consistent with the postulate that the primary process in the absence of the peroxide is



and in the presence of the peroxide is predominantly

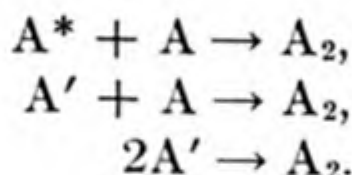


At temperatures at which the thermal reaction can be neglected, the overall process is a short chain reaction. It seems very probable that  $\dot{\text{R}}$ ,  $\text{RO}\dot{\text{O}}$ ,  $\dot{\text{H}}$ , and  $\text{H}\dot{\text{O}}_2$  are important intermediates in this process. Since the quantum yield of the reaction is inversely proportional to the square root of the intensity of the absorbed light, the chain-breaking step must be a bimolecular reaction between chain carriers (i.e., intermediates) leading to the production of stable molecules.



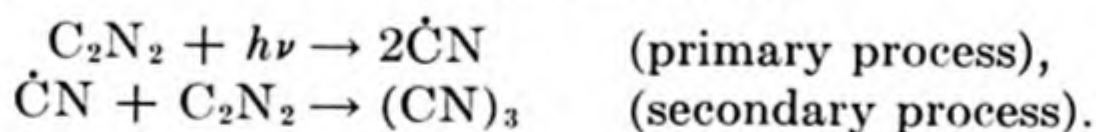
## POLYMERIZATION AND DIMERIZATION

There are two general types of polymerization: simple reactions leading to the formation of definite molecules, such as dimers or trimers, and chain reactions, whose products are macromolecules of indefinite molecular weight. One of the first polymerizations to be studied with reasonable care (Luther and Weigert, 1905) is the dimerization of anthracene. At moderately elevated temperatures (80°–200°C) the dimer reaches a measurable steady-state concentration in a dilute solution of anthracene illuminated with ultraviolet light (wave length 3660 or 3130 Å). Over the range of intensities and concentrations studied, the steady-state concentration is directly proportional to the intensity of the absorbed radiation. The quantum yield of dimerization increases with increasing anthracene concentration, approaching a limiting yield of about 0.5 (Weigert, 1927). The available measurements are insufficient to determine the mechanism of the process. It appears very probable that the first five steps which were proposed in the discussion of the oxidation of aromatic hydrocarbons occur in the polymerization reaction. However, any of the three following alternative reactions may be responsible for the formation of the dimer:



The last of these is consistent with the observed limiting yield of  $\frac{1}{2}$ . This interesting reaction is certainly worthy of further study.

The gas-phase polymerization of cyanogen (Hogness and Ts'ai, 1932) is at least superficially simple. The absorption of this compound is fairly strong in the wave-length region 2150–2250 Å. The absorption bands are diffuse, and the gas is nonfluorescent. Under the conditions of measurement the quantum yield is 3.0. The product is a brownish solid. The authors propose the following mechanism:



This is probably an oversimplification, since it is difficult to understand why the  $(CN)_3$  molecules would react with themselves to form paracyanogen but would not react with the remaining cyanogen.

The formation of large polymer molecules (Mark and Raff, 1941; Bawn, 1948) may occur either by successive condensation or by addition of simple molecules. Addition polymerization is essentially a chain reaction and, as such, can be studied effectively by photochemical methods. Determinations of the chain lengths of thermal reactions can be made only indirectly, usually by the use of inhibitors (Alyea and Bäckström, 1929). In a photochemical reaction the ratio of the over-all quantum

yield to the yield of the primary process (which is commonly close to unity) is a direct measure of the average chain length. Furthermore, knowledge of the nature of the primary product is frequently very helpful in the prediction of the secondary steps.

The primary act in association polymerization is the formation of two radicals or a diradical. Each radical or diradical can then add to a monomer molecule, forming a new radical of greater molecular weight. Large polymers are built up by the successive addition of monomer molecules to the growing radical. In most cases studied, the addition of monomer to the radical requires a heat of activation of a few kilocalories. The specific rate of addition is only slightly influenced by the size of the radical. In the absence of inhibitors the chain is, in the great majority of cases, terminated by a reaction between two radicals. This chain-stopping step eliminates two radicals either by their disproportionation or by their addition.

Free radicals may be formed by the photochemical dissociation of the monomer or of an added sensitizer such as acetone (Jones and Melville, 1946). Since the chains are broken by bimolecular reactions between growing radicals, the rate of polymerization is proportional to the square root of the intensity of the absorbed light.

A determination of the ratio of the rate constants for the chain-propagating and chain-terminating steps may be made by analyzing the kinetics of a polymerization reaction. This analysis is made by the usual steady-state approximate method. Special methods are required to evaluate either of these individual constants. Melville (1947) has shown that these individual constants can be obtained if the polymerization occurs under intermittent illumination. This technique, which has proved very useful in the study of polymerization kinetics, is a relatively old one in photochemistry, having been used by Berthoud and Bellenot in 1924 and subjected to a thorough theoretical analysis by Dickinson (Noyes and Leighton, 1941, pp. 202-209).

### INTRAMOLECULAR CHANGES

Relatively few photochemical isomerizations have been studied quantitatively. One group of reactions which has received some attention is the *cis-trans* isomerizations. For reasons of experimental convenience, most of the kinetic measurements have been made with substituted ethylenes. However, knowledge of their spectroscopic properties is limited to the simpler compounds. Figure 1-3, which is taken from the work of Mulliken (1942), is a schematic representation of the electronic energy levels of ethylene. In addition to the ground level *N*, two excited singlet levels, *V* and *R*, are shown. Absorption bands, corresponding to transition from *N* to either *V* or *R*, are strong. According to the Franck-Condon principle, the angle between the hydrogens cannot change appre-



ciably during the electron transition, and therefore the energies corresponding to transitions  $N \rightarrow R$  and  $N \rightarrow V$  will overlap. Transitions from  $N$  to  $R$  result in sharp bands, and from  $N$  to  $V$  in diffuse general absorption.

The optical and photochemical properties of *cis*- and *trans*-stilbene were carefully investigated by Lewis *et al.* (1940). *Trans*-stilbene is fluorescent (wave length 3300 to 4400 Å), and its absorption spectrum (wave length 2600 to 3400 Å) shows distinct "oscillational" structure; *cis*-stilbene is nonfluorescent and its absorption spectrum is apparently structureless. Irradiation of either pure compound with radiation of

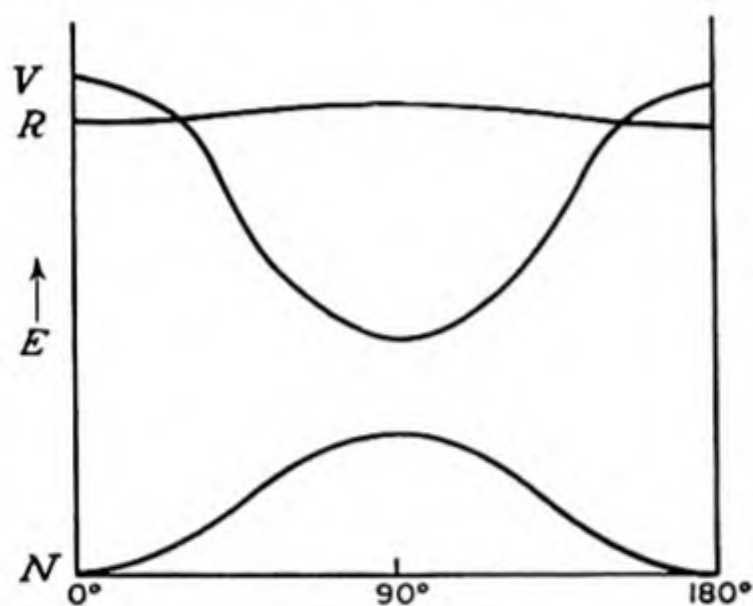


FIG. 1-3. Schematic potential-energy diagram for ethylene.

wave length 2537 Å produces partial stereoisomerization. Since *cis*-stilbene undergoes a photochemical side reaction to an unknown product, quantum yields had to be based on measurements of the initial rates. Starting with the pure *cis*-compound, the quantum yield of *trans*-stilbene formation is 0.26 and of the side reaction is 0.10. The corresponding quantum yield for the formation of *cis*- from *trans*-stilbene is 0.35. The interpretation of these facts is rendered uncertain by the lack of knowl-

edge of the potential-energy diagram for stilbene. The steric interference between the phenyl groups, which is responsible for the relative instability of the *cis*-form, undoubtedly renders the potential-energy curve for the ground state unsymmetrical and probably has a similar effect on the curves for the excited states. Conjugation between the benzene rings and the ethylenic link must also affect the energy levels. For lack of other information, let us assume that the potential-energy curves for stilbene, although asymmetric, are otherwise essentially similar to those for ethylene. A molecule excited to the state  $V$  will quickly lose its extra energy of oscillation by successive impacts with solvent molecules and will end up in the (approximately) 90° trough of the electronic state. The subsequent transitions of the molecule are, of course, independent of whether it was originally a normal *cis*- or *trans*-stilbene molecule. Since transitions between states  $N$  and  $V$  are permitted, it might be expected that the excited molecule could emit a quantum and return to the ground state of either the *cis*- or *trans*-form, depending on the relative asymmetries of states  $N$  and  $V$ . If this were the mechanism of the process, the quantum yields of fluorescence and isomerization would not be complementary, and the limit of the sum of these yields would be 2 rather than 1. That this mechanism does not apply to this case is shown by the nonfluorescence of *cis*-stilbene. The strong fluorescence of the

*trans* compound demonstrates that some (or all) of these excited molecules are in a state which is peculiar to the *trans*-configuration, possibly state *R*. If *cis*-stilbene is excited to state *R* it must go, by internal conversion, to state *V* (or possibly to the ground state with a high excess of oscillational energy) in a time much less than the natural half life of the excited state. The fluorescent yield of *trans*-stilbene was not measured. Lewis and his coworkers (1940) assumed that the ratio of the nonradiative return to the *cis*- and *trans*-forms was independent of whether the excited state was formed by the irradiation of normal *cis*- or *trans*-stilbene. This assumption leads to a value of about 0.5 for the fluorescent yields. Although this latter assumption is consistent with the available data, it is by no means the only reasonable interpretation. Olson's conclusion (1931) that the compound formed from the excited state will be predominantly the isomer of lower stability should not be expected to apply to a reaction which takes place by way of internal conversion. The probability of such a process will depend on the relative forms of the several potential-energy surfaces and on their points of intersection and not merely on relative times spent in the two rotational configurations. Photochemical reactions of this type deserve much more attention than they have received. They are intrinsically interesting, and an understanding of them should prove helpful in the interpretation of more complex photochemical processes (Pinckard *et al.*, 1948; Stearns, 1942).

The photoisomerization of *o*-nitrobenzaldehyde to *o*-nitrosobenzoic acid involves the breaking of two bonds and the formation of two new ones, but the reaction appears to be strictly an intramolecular process. The course of the reaction is independent of whether the compound is present as crystals, is in solution in a solvent, e.g., acetone, or is in the vapor phase. No detectable oxygen is liberated. In the condensed systems (Leighton and Lucy, 1934) at room temperature the quantum yield is  $0.50 \pm 0.03$ . In the vapor phase at 90°C, where the vapor pressure is about 4 mm of Hg, the quantum yield is  $0.70 \pm 0.05$ , but the yield is reduced by the addition of molecular nitrogen, reaching a value of about 0.5 at a nitrogen pressure of 700 mm of Hg (Küchler and Patat, 1936). The yield in solution for the corresponding reaction of 2,4-dinitrobenzaldehyde is also about 0.5 but is approximately 0.7 for 2,4,6-trinitrobenzaldehyde. It is plausible that the reaction involves the hydrogen-bonded quasi six-membered ring and that it takes place by way of an act of internal conversion. Why the yield reaches a limiting value of 0.5 in condensed systems or in the presence of an inert gas is not obvious. The theoretical predictions of Leighton and Lucy are incompatible with the subsequent experiments of Küchler and Patat, and therefore this detailed theory apparently must be rejected.

The photochemical denaturation of proteins and inactivation of enzymes can be classed, somewhat arbitrarily, as rearrangements of the



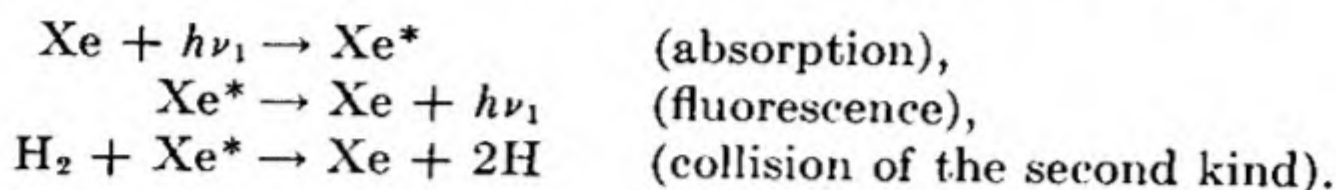
hydrated protein molecules. These reactions have been studied extensively in recent years (McLaren, 1949), and some empirical generalizations can be deduced from the results of these studies. The quantum yields of the reactions are in the range from  $10^{-2}$  to  $10^{-4}$ . Radiation of wave lengths shorter than 3100 Å is required to produce the reactions. Photochemical denaturation is irreversible. The primary photochemical product remains in solution at low temperatures (e.g., 4°C), but a precipitate forms rapidly when the previously irradiated solution is heated to 40°C. The quantum yield of the primary process is practically independent of temperature over the narrow range available. Photochemical denaturation results from the irradiation of "dry" proteins as well as proteins in dilute aqueous solution. The quantum yield is not a function of the intensity of the absorbed light.

If the quantum yield is strictly independent of the intensity of the light absorbed by the native protein, the primary act involves the interaction of one photon with each molecule; i.e., it is a "single-hit" process, in which there is no cooperative action between two or more photons either successively or simultaneously. It should be realized, however, that no simple mechanism predicts that the yield is independent of the product of incident intensity and time of irradiation since the (dissolved) denatured protein must act as an efficient internal filter. The "one-hit" kinetics have been interpreted in terms of a primary act in which one peptide linkage is broken by a single absorbed photon. The low quantum yields indicate that this primary process is very inefficient; most of the absorbed quanta are degraded to heat. One plausible explanation for the observed inefficiency is that the light, which is absorbed by aromatic nuclei in the molecule, becomes available for chemical action by an act of internal conversion. A few facts which support this tentative explanation are (1) the yield increases with increasing frequency (i.e., energy) of the photon, (2) the yield decreases with increasing size of the molecule, and (3) the yield is greater for adsorbed films of proteins than it is for solutions.

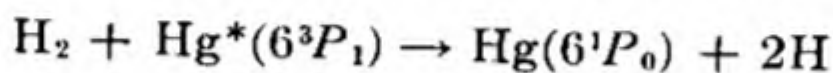
### SENSITIZED REACTIONS

In sensitized reactions the substance which absorbs the light does not undergo any permanent chemical change. This absorbing substance, called the "sensitizer," catalyzes the photochemical reaction. The simplest known example of this type is the xenon-sensitized photochemical dissociation of hydrogen (Calvert, 1932). The resonance radiation of xenon has a wave length of 1469 Å, which corresponds to an energy of 193 kcal/mole. Molecular hydrogen, whose dissociation energy is 103 kcal/mole, does not absorb radiation of wave length longer than 849 Å. If a mixture of xenon and hydrogen is illuminated with a xenon arc, hydrogen atoms are formed as was demonstrated by their color reaction with

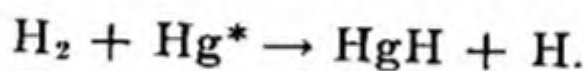
solid tungstic oxide. If pure hydrogen is substituted for the mixture of gases, there is no reaction. The reaction steps are as follows:



Mercury vapor is a better-known sensitizer for the dissociation of hydrogen (Noyes and Leighton, 1941). The first resonance radiation of mercury, wave length 2537 Å, corresponds to an energy of 112 kcal/einstein, which is only slightly more than is necessary to dissociate molecular hydrogen. It should be expected that the reaction

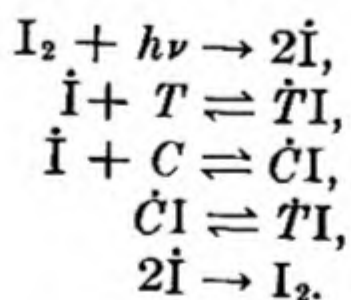


should be very efficient. Although the interaction between an excited mercury atom and a hydrogen molecule is indeed very probable, HgH appears to be one of the products:



If other reactant gases, such as carbon monoxide or ethylene, are present, the hydrogen atoms initiate a series of reaction steps leading to a variety of products. Many such mercury-sensitized reactions have been studied.

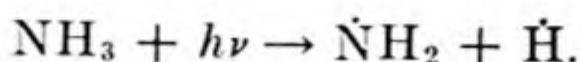
Photochemical *cis-trans* isomerizations are sensitized by iodine (Berthoud and Urech, 1930; Dickinson *et al.*, 1949). The sensitized reaction is a short chain process with an appreciable heat of activation. The primary act is the photodissociation of molecular iodine. Iodine atoms can add, with an appreciable heat of activation, to carbon atoms adjacent to the double bond. This opens the double bond, permitting rotation of the groups. Subsequently the iodine atom can split off. This mechanism is summarized in the following equations, in which *C* and *T* stand, respectively, for the *cis* and *trans* form of the molecule:



In some systems an absorbing compound, undergoing a permanent photochemical reaction with a yield of about 1, simultaneously induces a chain reaction between other reactants. The chain reaction so overshadows the inducing reaction, that the whole process may be thought of, loosely, as a sensitized photochemical reaction. Examples of this type are the oxidation of hydrogen (Farkas *et al.*, 1930) and the polymerization



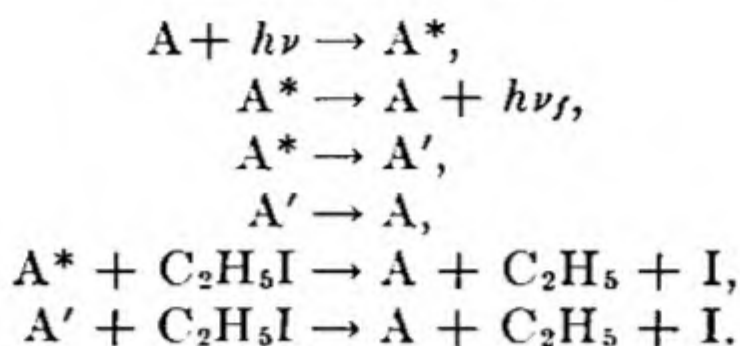
of ethylene (Taylor and Emelius, 1931) induced by the predissociation of ammonia:



When a mixture of hydrogen, oxygen, and ammonia, at a moderately elevated temperature, is illuminated with light of wave length 2200 Å or shorter, ammonia is decomposed and water is formed. The quantum yield for the formation of water increases from about 25 at 290°C to approximately 380 at 405°C. At 420°C, irradiation of the system results in an explosion. The kinetics are complex (Lewis and von Elbe, 1938) and probably involve the amide radical as well as the hydrogen atom.

If a mixture of carbon monoxide and chlorine is illuminated with light which is absorbed by the chlorine, a chain reaction ensues, the product of which is phosgene. The kinetics of the reaction are complex, but the primary act is certainly the dissociation of chlorine and the radicals  $\dot{\text{Cl}}$  and  $\dot{\text{COCl}}$  are involved in the secondary reactions. If an excess of oxygen is added to the system, the formation of phosgene is suppressed, and the predominant process becomes the sensitized formation of carbon dioxide (Rollefson and Burton, 1939, pp. 313–319). The quantum yield of carbon dioxide formation is large and is a complex function of temperature and the partial pressures of the reactants. A number of reactions of this general type have been studied, but the mechanism of none of them is completely understood.

The photolysis of ethyl iodide is sensitized (West and Miller, 1940; West, 1941) by naphthalene and a number of its derivatives. The direct photolysis of ethyl iodide occurs both in the gas phase and in solution. In hexane solutions the quantum yields corresponding to wave lengths 3130 and 2537 Å are about 0.30 and 0.40, respectively. The quantum yield of the naphthalene-sensitized process is about 0.30 for either wave length. The maximum fluorescence efficiency of naphthalene in hexane solutions is approximately 0.15. As was clearly stated by West (1941), this demonstrates that ethyl iodide can interact with a nonfluorescent excited state as well as with the fluorescent excited state of the naphthalene molecule. The yield of the sensitized reaction is independent of the naphthalene concentration but falls off to small values when the ethyl iodide concentration is decreased much below  $10^{-2} M$ . These results, as well as observations on the effect of changing the viscosity of the solvent, show that the sensitization is a collisional process, that the efficiency of such collisions in producing the reaction is high (probably greater than 0.1), and that the collisions in the condensed system occur in bursts (p. 15). All these data are consistent with the following mechanism, which is strikingly similar to the mechanism here offered as an explanation (p. 28) for the photoautooxidation of aromatic hydrocarbons (Bowen and Williams, 1939):



Photoautooxidations of reactive reducing agents are sensitized by a wide variety of dyes and pigments (Hurd and Livingston, 1940). These reactions occur in aqueous solutions or in organic solvents, such as methanol or acetone. In cases where the kinetics have been studied in detail, they appear to be complex, and in no case has a completely satisfactory mechanism been proposed. These reactions are produced by either visible light or ultraviolet radiation, depending chiefly on the absorption spectra of the sensitizers.

Sensitized photochemical redox reactions are of great importance in biology, the outstanding example being photosynthesis by green plants (Rabinowitch, 1945). Photodynamic action and certain pathological skin reactions (Blum, 1941) are also of interest. The oxidative inactivation of enzymes is sensitized by riboflavin (Galston and Baker, 1949) as well as by certain dyes.

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## CHAPTER 2

# Practical Applications and Sources of Ultraviolet Energy

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*Introduction. Germicidal-action curves: Action curve tentative at shorter wave lengths—Action curve approximate at longer wave lengths—The unique 2537 Å mercury line. Susceptibility to ultraviolet: Injury, mutation, and kill—Comparison of susceptibility to ultraviolet and to other lethal agents—Logarithmic nature of kill—Unit kill—Reactivation by heat and light—Germicidal action of ultraviolet of wave lengths greater than 2800 Å—Composite of killing factors. Killing exposures: Reciprocity of time and intensity. Erythematous action of wave lengths 2537 and 2967 Å: American Medical Association tolerance—Face and eye protection and treatment. Commercial sources of ultraviolet: High- and low-pressure mercury arcs—Ultraviolet of wave length 2537 Å—Conversion factors—Intensity—Ozone formation—Photochemical effects of 2537 and 1849 Å energy—Temperature and ventilation—Depreciation. Ultraviolet disinfection: Air disinfection—Fluid disinfection—Disinfection of surfaces of granular materials. Ultraviolet-induced mutants for new fungi. Protection and processing of products: Mold, antibiotics, and parenteral fluids—Blood plasma—Syrup, fruit-juice, and wine storage—Meal storage. Higher pressure mercury sources of ultraviolet: Intensity and variations with distance—Individual line intensities—Starting and restarting times—Life and depreciation—Research determination of output and intensity—Mercury-amalgam and other metal arcs—Sunlamps. References.*

## INTRODUCTION

Innumerable applications of ultraviolet energy are suggested in a voluminous amount of old literature, in which there is little of practical value because of the failure to specify the ultraviolet wave lengths, the intensities, and the exposure times used. This is equally true of the many chemical, the indefinite therapeutic, and the few biological effects of the ultraviolet. Ellis *et al.* (1941) have comprehensively reviewed the chemical and biological applications of the ultraviolet; Laurens (1933) has done the same for the physiological effects. Meyer and Seitz (1949) and Koller (1952) have excellently reviewed the sources, measurement, and various applications of the ultraviolet. Lea (1946) has contrasted the excitation effects of ultraviolet with the ionization effects of X ray and shorter wave length radiations in a practical discussion of the theoretical bases of both effects.

The practical biological applications of ultraviolet are those utilizing its erythral effects, its ergosterol activation, and its inactivating and mutational effects on bacteria, fungi, and viruses. The action spectra describing all these effects as functions of wave length are subjects of other chapters; this chapter is concerned with one outstandingly practical biological application, the germicidal effect. There are included discussions of commercially available sources of ultraviolet for this effect and for research on this and other effects.

### GERMICIDAL-ACTION CURVES

If bacteria are irradiated with ultraviolet of various wave lengths and with an identical exposure (intensity times time) for each wave length,

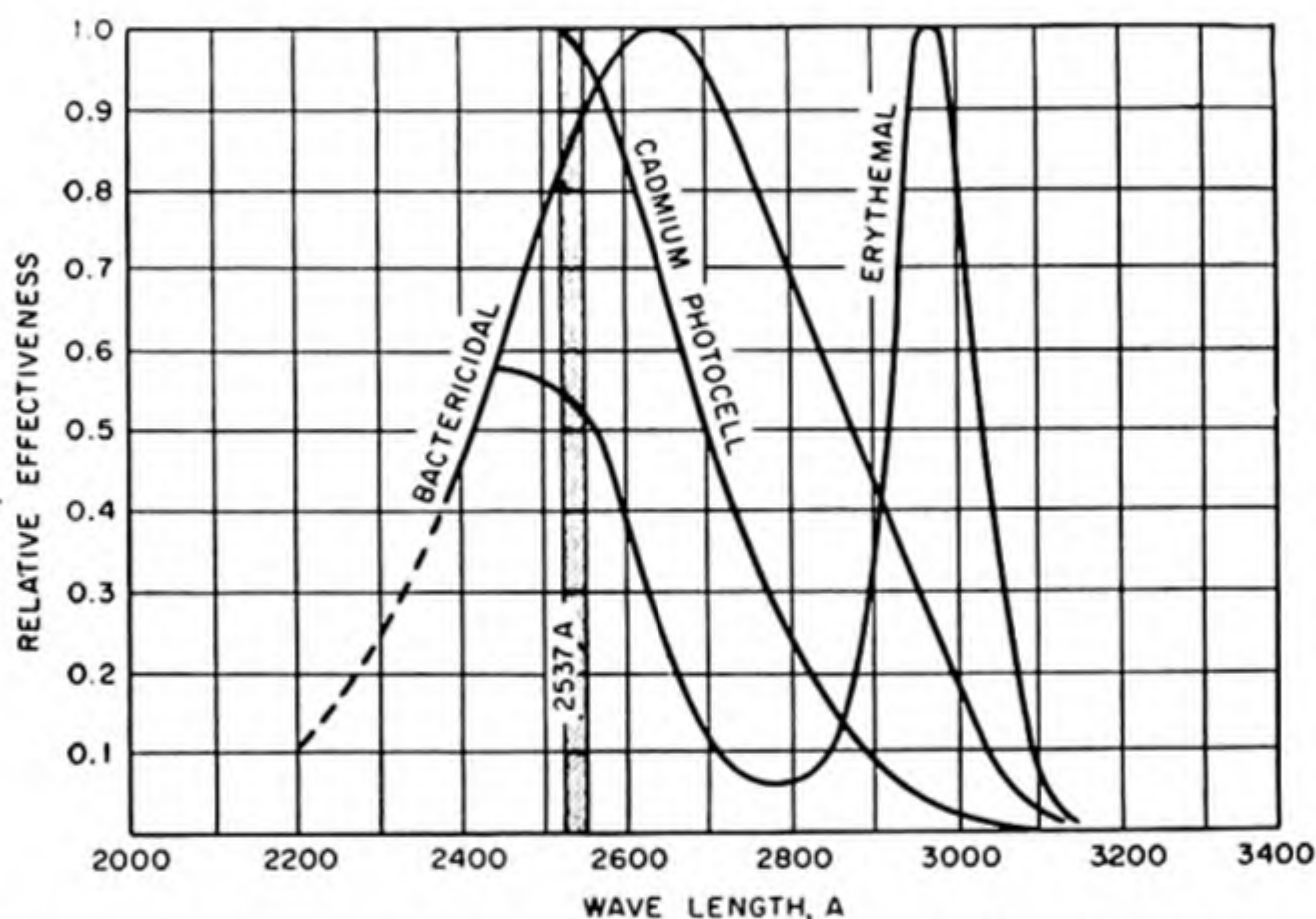


FIG. 2-1. Bactericidal- and erythral-action curves. (*International Commission on Illumination, Berlin, 1935.*)

which is sufficient to give a convenient unit of killing, 50, 63.2 (lethe; see p. 49), or 90 per cent, at the optimum wave length, the data may be plotted as a germicidal-action curve. Such a curve has not been standardized as have the curves for luminosity and the erythral action, but Gates (1929-30), Hollaender *et al.* (1940), Jones *et al.* (1940), and others have studied the action of specific wave lengths on specific organisms. Caspersson (1931, 1937) associates the germicidal-action curve with the ultraviolet-absorption curve of the nuclear protein. For most bacteria and fungi and for some viruses, the optimum killing wave length is at  $\sim 2650$  Å. The relative effects at longer and shorter wave lengths are so similar that a single tentative action curve for the average germicidal effect on various bacteria and fungi and on many viruses is shown in Fig. 2-1 along with an erythral-action curve standardized by the Inter-



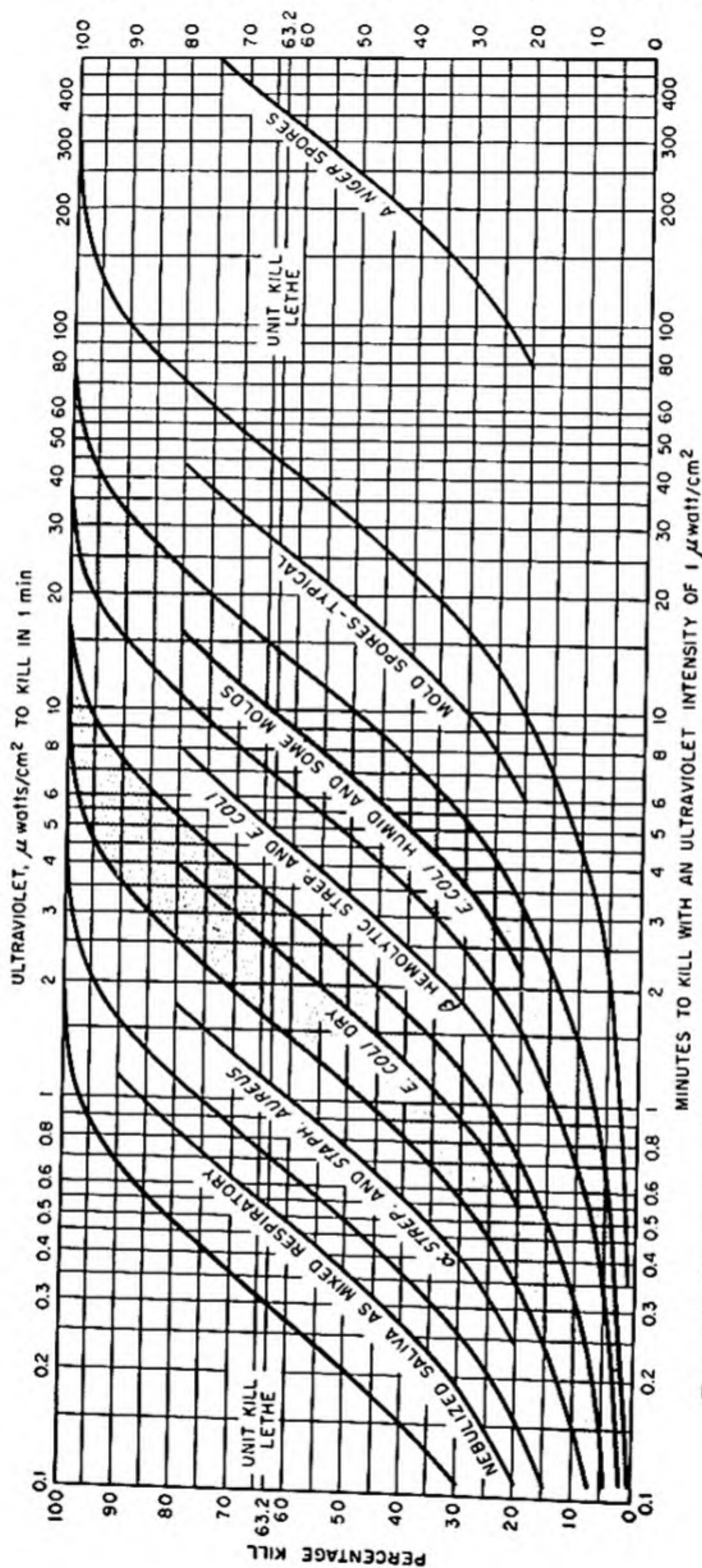


Fig. 2-2. Exposures (intensity times time) for various degrees of killing of various microorganisms.



national Commission on Illumination (ICI), Berlin (1935). Both curves are plotted with the relative effect at the optimum wave length as 100 per cent. The relative 100 per cent bactericidal effectiveness of 2650 Å and the 85 per cent relative effectiveness of 2537 Å energy shown in Fig. 2-1 should not be confused with the possibility of a 100 per cent absolute kill by 2537 Å energy shown in Figs. 2-2 and 3. The ICI factors for the erythema-action curve and tentative factors for the bactericidal-action curve proposed in Fig. 2-1 are shown for individual mercury lines in Table 2-1. These factors are useful for calculating the relative effectiveness and

TABLE 2-1. ERYTHEMAL- AND BACTERICIDAL-ACTION FACTORS

Mercury spectrum lines, Å	ICI erythema factors	Tentative bactericidal factors
2353	0.55	0.35
2446	0.57	0.58
2482	0.57	0.70
2537	0.55	0.85
2576	0.49	0.94
(2650)	....	1.00
2654	0.25	0.99
2675	0.20	0.98
2700	0.14	0.95
2753	0.07	0.81
2804	0.06	0.68
2857	0.10	0.55
2894	0.25	0.46
2925	0.70	0.38
2967	1.00	0.27
3022	0.55	0.13
3130	0.03	0.01

efficiency of mercury sources whose relative line intensities are known (see Tables 2-3 and 6).

#### ACTION CURVE TENTATIVE AT SHORTER WAVE LENGTHS

The germicidal-action curve for wave lengths less than 2500 Å is still tentative since theory and some research suggest that the action continues to increase at shorter wave lengths (greater frequencies and greater energy content of the quanta). A rather rapid drop in the curve at wave lengths less than 2500 Å is, on the other hand, characteristic of the absorption curve of nuclear protein and very representative of practical germicidal effects in which nearly all the liquid and gaseous elements in the environment of an organism absorb the ultraviolet of shorter wave lengths and

thus protect the organism itself. Perhaps the decreasing germicidal action at wave lengths less than 2500 Å found by some workers may represent the absorption curves of the culture media rather than the absolute susceptibility of naked bacteria to ultraviolet killing.

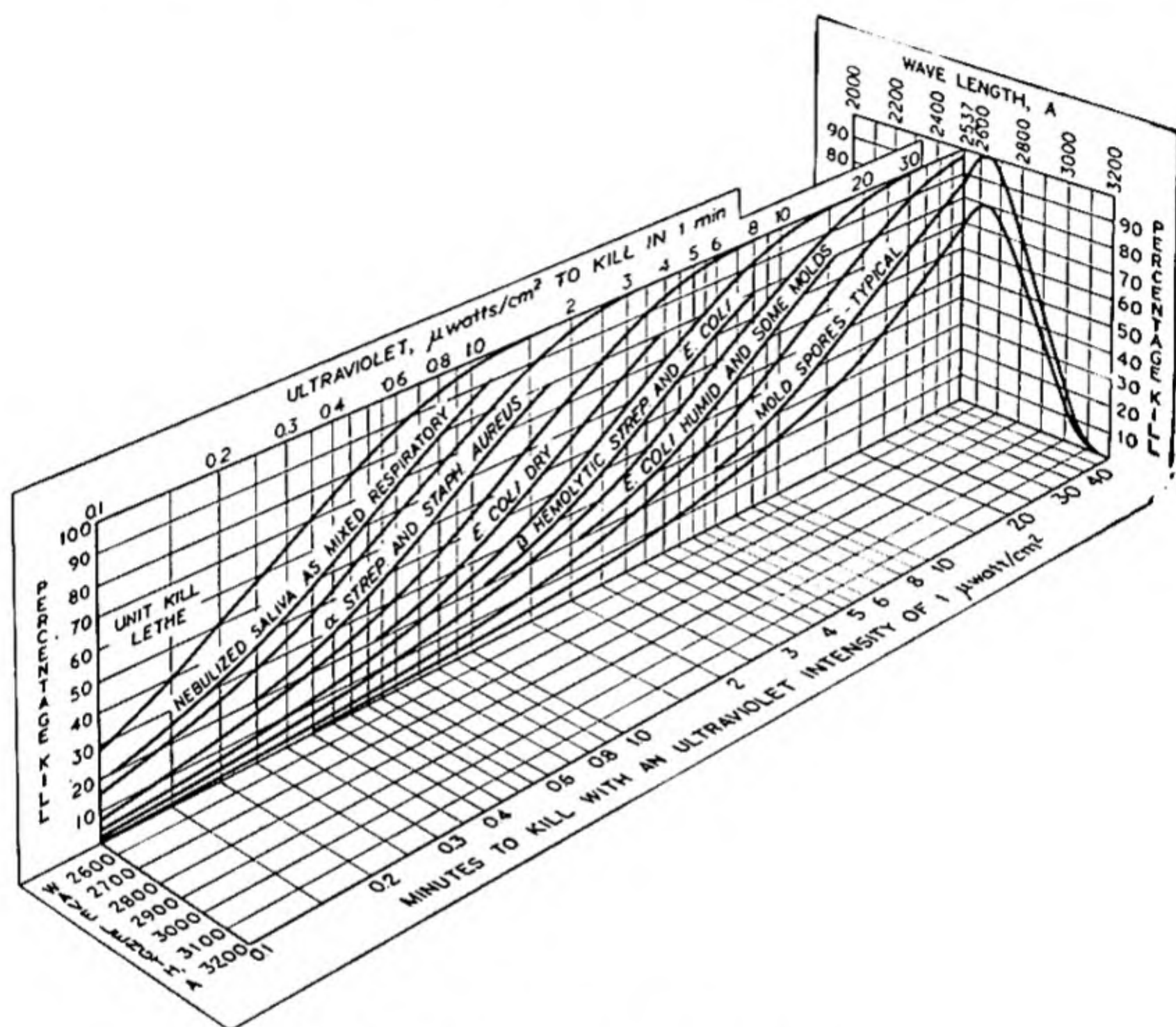


FIG. 2-3. Composite of Figs. 2-1 and 2.

### ACTION CURVE APPROXIMATE AT LONGER WAVE LENGTHS

The germicidal action of radiant energy extends even into the visible spectrum, the action decreasing rapidly with increasing wave length (decreasing frequency and energy content of quanta). The action is of the order of magnitude in the near ultraviolet and visible estimated by Luckiesh and Taylor (1946; see also Hollaender and Claus, 1935-36) in Fig. 2-4 where a logarithmic ordinate is used to extend the killing action to lower effectiveness levels. The reactivating effects of wave lengths 3600-4400 Å reported by Kelner (1949) suggest that, for all practical purposes, the curve of Fig. 2-4 might well end at 3600 Å. In any case, since



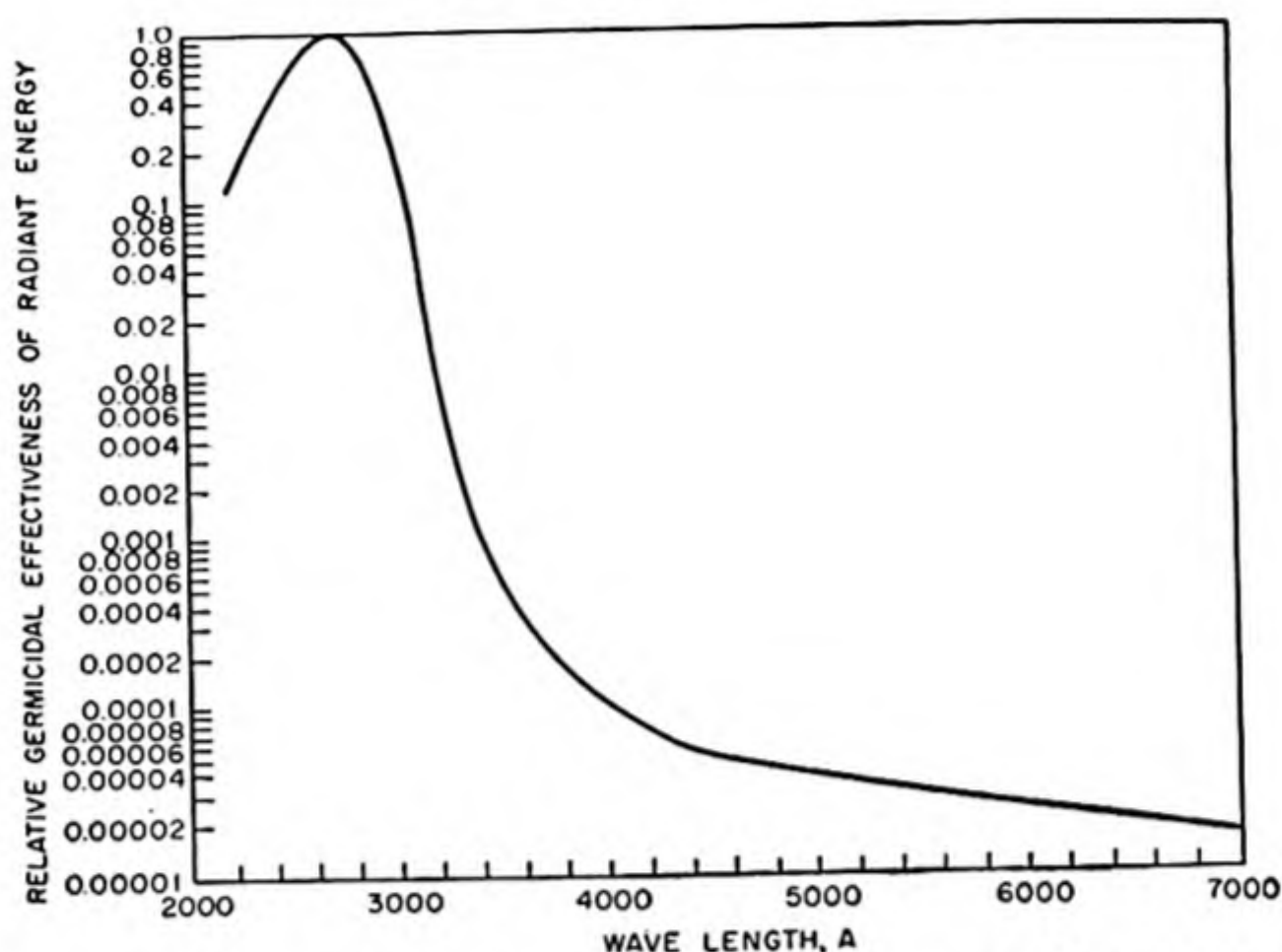


FIG. 2-4. Relative bactericidal action extended to the near-ultraviolet and visible regions for *E. coli* on agar. (Luckiesh *et al.*, 1947; Hollaender and Claus, 1935-36.)

no effects on fungi are reported, the curve probably applies only to the more susceptible organisms, at wave lengths greater than 3600 A.

### THE UNIQUE 2537 A MERCURY LINE

Sixty per cent of the electrical input to a low-pressure mercury arc is converted directly into radiation of wave length 2537 A. Wave length 2537 A produces 85 per cent of the maximum germicidal effect on most bacteria, fungi, and viruses which is possible at  $\sim 2650$  A. This efficient production of ultraviolet of nearly optimum germicidal wave length is one of the more unusual coincidences in biophysics. The shape of the germicidal-action curve is such that the effectiveness of 2537 A ultraviolet is only 10-20 per cent less than the maximum effectiveness possible at 2650 A, an uncertainty well within the variations in the action curves for various organisms and within the experimental errors inherent in the determination of the curves. For these reasons the low-pressure mercury arc has been selected as the practical source of ultraviolet for germicidal effects. About the only practical interest in the germicidal-action curve is to appraise the relative inefficiency of high-pressure mercury arcs in fused quartz glass, the only artificial sources of ultraviolet that are at all comparable with the low-pressure arcs. In Fig. 2-5 the action curves are superposed on block diagrams of the relative line intensities of typical high- and low-pressure mercury arcs. The block diagrams are calculated on the basis of equal amounts of power (in watts) into the two types of arcs in order to show graphically their relative ultraviolet efficiencies as well as their germicidal and erythematous effectiveness.



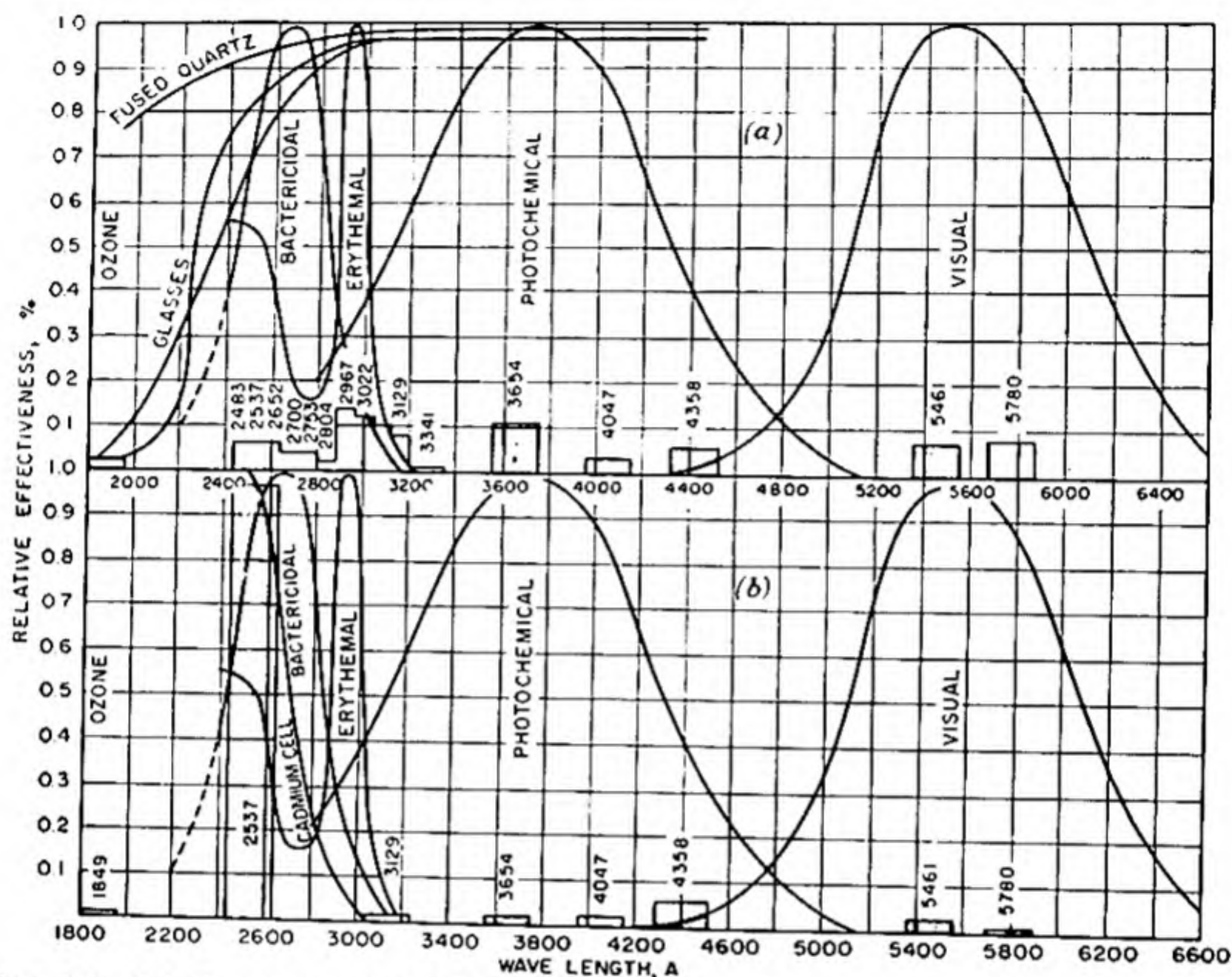


FIG. 2-5. Relative energy distribution of (a) high- and (b) low-pressure mercury arcs, transmission of arc tube glasses, and action curves.

### SUSCEPTIBILITY TO ULTRAVIOLET INJURY, MUTATION, AND KILL

An understanding of the biophysical nature of cell injury, mutation, and kill is not essential for the practical applications of the ultraviolet. In this discussion, for practical purposes, an organism is considered dead when it is unable to reproduce. The possibility of this being a condition far short of complete destruction, and the logarithmic nature of ultraviolet killing, leave considerable uncertainty as to just when all the organisms in one group are dead. In most of the practical ultraviolet applications a complete kill is not necessary, but whenever it is necessary it can be provided by adequate factors of certainty in exposure.

There is ample evidence from practical experience that the growth of fungi can be prevented by exposures of the order of those effective for bacteria killing and by exposure of only a fraction (a tenth to a hundredth) of those required for the killing of bacterial spores. The wilting and death of common plants such as ivy and tomato under such exposures sug-

gest suppression of the mycelia without serious damage to the spores, but the mechanism should be an interesting subject for research.

### COMPARISON OF SUSCEPTIBILITY TO ULTRAVIOLET AND TO OTHER LETHAL AGENTS

The intrinsic susceptibility of various species of bacteria, fungi, and viruses to killing by ultraviolet of 2537 Å, or any other wave length, varies over an exposure range of as much as 1-3. The extrinsic susceptibility, determined by acquired tolerance and age, may also vary over a range of 1-3. In contrast with bacteria and viruses, for any arbitrary percentage kill of various mold spores, the exposure may be 15-300 times that required for the same kill of dry air-borne *Escherichia coli*, with a difference as great as 1 to 1000 between the most susceptible bacteria and the most resistant mold spores. The exposure necessary to kill any one kind of organism may vary considerably, depending on its environment, temperature, illumination, and physical condition, illustrated in Fig. 2-2 by humid water-borne *E. coli* which requires four times the exposures of dry air-borne *E. coli* for comparable killings.

Although the effects of radiation of shorter wave lengths on various microorganisms seem to be very similar to those of the ultraviolet, there is little similarity between the effects of ultraviolet and of other lethal agents such as heat, dryness, or chemicals except in the logarithmic nature of the kill. Markedly thermoduric organisms, for example, are readily killed by ultraviolet energy.

### LOGARITHMIC NATURE OF KILL

The practical ultraviolet killing of nearly all microorganisms is more or less logarithmic in nature. Wyckoff (1932) and Rahn (1932, 1945) have discussed this rule and its exceptions in detail. The logarithmic nature of kill is in accord with the general exponential attenuation law,

$$N = N_0 e^{-It}, \quad (2-1)$$

where

$N_0$  = the initial concentration of organisms,

$N$  = concentration of organisms after an exposure for time  $t$  to an ultraviolet intensity,

$I$  = the ultraviolet intensity, and

$e$  = the base of the natural logarithms (about 2.718).

For a unit exposure,  $It = 1$ , the concentration of survivors  $N$  becomes

$$N = N_0 e^{-1} = 0.368 N_0, \quad (2-2)$$

and the kill,  $N_0 - N$ , becomes

$$N_0 - N = 0.632 N_0. \quad (2-3)$$



The same attenuation law also covers the dilution of fluid-borne contamination by the admixture of a neutral and sterile fluid when the unit volume of diluent is substituted for  $I$  in the exponent.

### UNIT KILL

This relation suggests 63.2 per cent as a basic unit of sanitation for which the term "lethe" has been suggested. With air sanitation in mind, Wells (1940) has also used the term for a unit ultraviolet exposure producing a 63.2 per cent kill of a standard organism (*E. coli*) under elaborately specified conditions. A lethal exposure then becomes equivalent

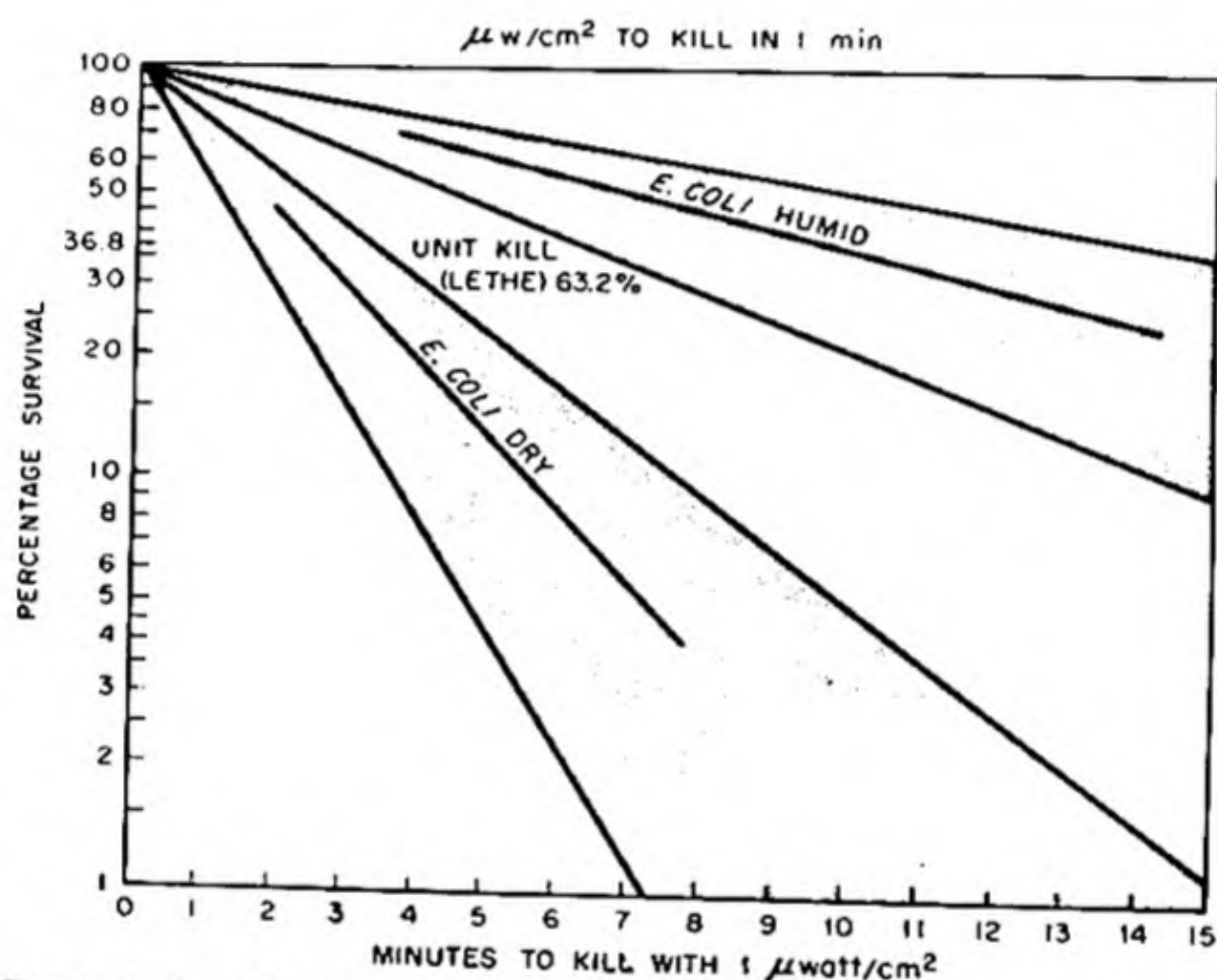


FIG. 2-6. Typical data from Fig. 2-2 shown on a logarithmic scale of percentage of survivors and uniform scale of exposures.

in effect to the air change of mechanical ventilation, as discussed later. The lethe unit of kill is indicated in Figs. 2-2 and 6.

The exponential form of the curve of killing as a function of exposure has also led to the suggestion of a unit kill of 50 per cent, by analogy with the half-life rating of radioactive materials.

The logarithmic nature of the ultraviolet germicidal effect is illustrated in Fig. 2-6 where the data on dry and wet *E. coli* are plotted on a logarithmic scale of survivors and on an arithmetic scale of exposures. Most of the published data plot as straight lines on such scales within the experimental errors of the measurements [see Lea (1946) for a discussion of methods of plotting such data]. An arithmetic or linear scale of kill is used in Fig. 2-2, along with a logarithmic scale to expand the range of



lesser exposures and yet cover a thousandfold exposure range. The sigmoid form of the curves is inherent in the scales used.

### REACTIVATION BY HEAT AND LIGHT

The practical significance of the reactivating effect of time, heat, and light on ultraviolet-injured bacteria and molds was overemphasized by the manner in which the experimental data were first presented by Kelner (1949). For example, a definite 20 per cent revival of the total initial number of irradiated bacteria per experimental unit volume was presented as a 3000 per cent increase in the number of viable organisms from the indefinite few left after a theoretical killing of 99.99994 per cent. By this method of presentation, if the "killing" had been complete, the percentage increase in viable organisms would have been infinite regardless of the actual revival.

The 20–25 per cent revivals of bacteria resulted from light exposures of the order of 5–8000 ft-c-hr, exposures provided only by 2–3 days of the highest levels of practical indoor illumination. The equivalent of a 2- to 3-hr exposure to 100 ft-c provided less than a 0.1 per cent revival, and few practical germicidal applications involve exposures of bacteria to as many foot-candle-hours. This suggests that the reactivating effect of light is of little or no significance indoors but may somewhat reduce the apparent susceptibility of organisms to the ultraviolet of the sun.

### GERMICIDAL ACTION OF ULTRAVIOLET OF WAVE LENGTHS GREATER THAN 2800 Å

Buchbinder *et al.* (1941) have shown that sunlight, direct and through window glass, as well as the ultraviolet from common artificial light sources, has measurable germicidal effects on bacteria exposed to common illumination intensities for a day or two (Fig. 2-4). The daylight intensities and exposure times may have been somewhat comparable with those used in the reactivation experiments, in which case the killing must have been the difference between the germicidal action of wave lengths greater than 3000–3200 Å and the reviving or protective action of wave lengths greater than 3600 Å.

### COMPOSITE OF KILLING FACTORS

Only a three-dimensional model would completely represent the relations of wave length and exposure to killing. However, the outstanding practicality of wave length 2537 Å suggested making its plane representative of the wave length and plotting on it a fourth indeterminate variable, the susceptibility of organisms to killing by that wave length, as typical of the killing by other wave lengths. The result was a consolidation of Figs. 2-1 and 2 as Fig. 2-3.

## KILLING EXPOSURES

## RECIPROCITY OF TIME AND INTENSITY

Like the photographic effects of light, the germicidal effect of ultraviolet results from an exposure (intensity times time). The basic factors in an exposure are the incident power, the time, and the irradiated area. The erg, often used in biophysical work, is a unit of energy only and must be referred to time in order to define power. Ergs per second become units of power, and ergs per second per square centimeter become units of intensity. Ergs per second per square centimeter-second, or, more usually, ergs per square centimeter, become units of dose or exposure. In practical work there are advantages in basing the intensity unit directly on the watt since it is also used to define the power output of ultraviolet sources. The microwatt, equal to 10 ergs/sec, becomes a convenient unit of power, and the microwatt per square centimeter becomes a convenient unit of intensity. For practical purposes the microwatts per square centimeter equal the milliwatts per square foot, the multiplying factor being 0.9290. The microwatt per square centimeter-minute, often written as microwatt-minute per square centimeter, becomes a practical unit of exposure equal to 600 ergs/cm<sup>2</sup>. The microwatts per square centimeter-minute emphasizes the reciprocity of exposure intensities and times which may be adjusted over a very wide range to obtain a specified exposure under various conditions.

Theoretically, an exposure of 25  $\mu\text{w-min/cm}^2$  or 1500 ergs/cm<sup>2</sup>, for example, may be obtained either in a long time (1 day) with a low intensity (0.018 ultraviolet  $\mu\text{w/cm}^2$ ) or in a short time (0.001 min) with a high intensity (25,000 ultraviolet  $\mu\text{w/cm}^2$ ). In practice, the exposure time is usually determined by the nature of the job to be done and ranges from a fraction of a second for the disinfection of rapidly moving air or products to 1-10 min for air disinfection in relatively quiet places. The exposure intensity must then be adjusted to obtain an adequate exposure. Such intensities may range from a few ultraviolet microwatts per square centimeter for bacterial air disinfection to several ultraviolet milliwatts per square centimeter for product disinfection from molds, as suggested by Fig. 2-7a,b and in greater detail for the short exposures in air ducts by Fig. 2-8.

The reciprocity of time and intensity is also illustrated by the upper and lower legends on the otherwise identical scales of exposure of Fig. 2-2.

## ERYTHEMAL ACTION OF WAVE LENGTHS 2537 AND 2967 A

As shown graphically in Figs. 2-1 and 5, the germicidal ultraviolet is also erythema in action, the effect at 2537 A being about half as great as at the optimum wave length of 2967 A. As indicated in Figs. 2-1 and 7a,



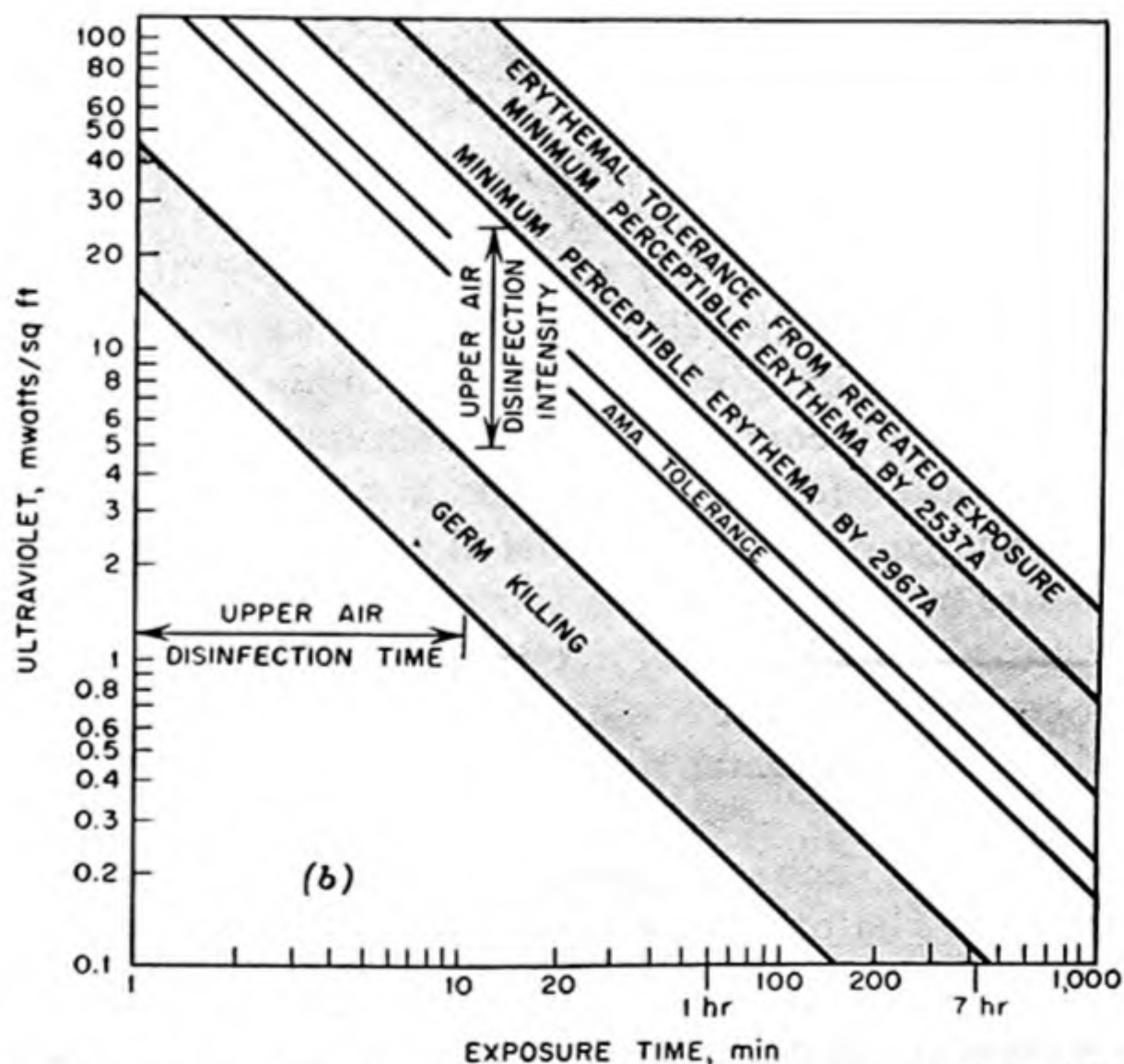
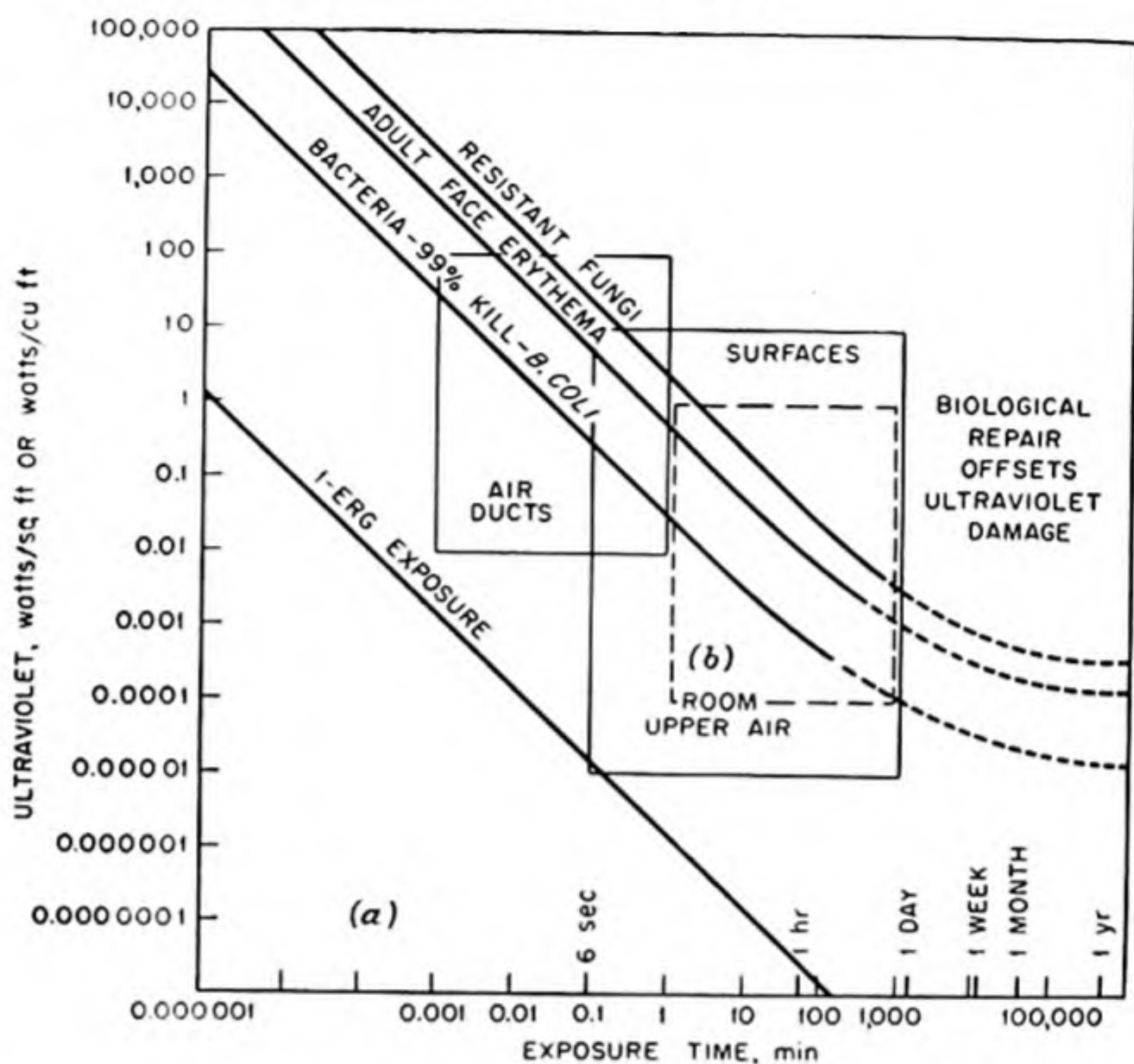


FIG. 2-7. Reciprocity of time and intensity for various exposures and the kill of typical microorganisms.



erythematous exposures are about 10 times the germicidal and are comparable with the fungicidal exposures, so that the time element becomes important in practical applications. Intensities, which are germicidal within the few seconds and minutes required, in many cases become erythematous with 10–15 times longer exposures of the face and eyes, thus making some form of protection usually necessary. As suggested in Fig. 2-7b, an exposure to

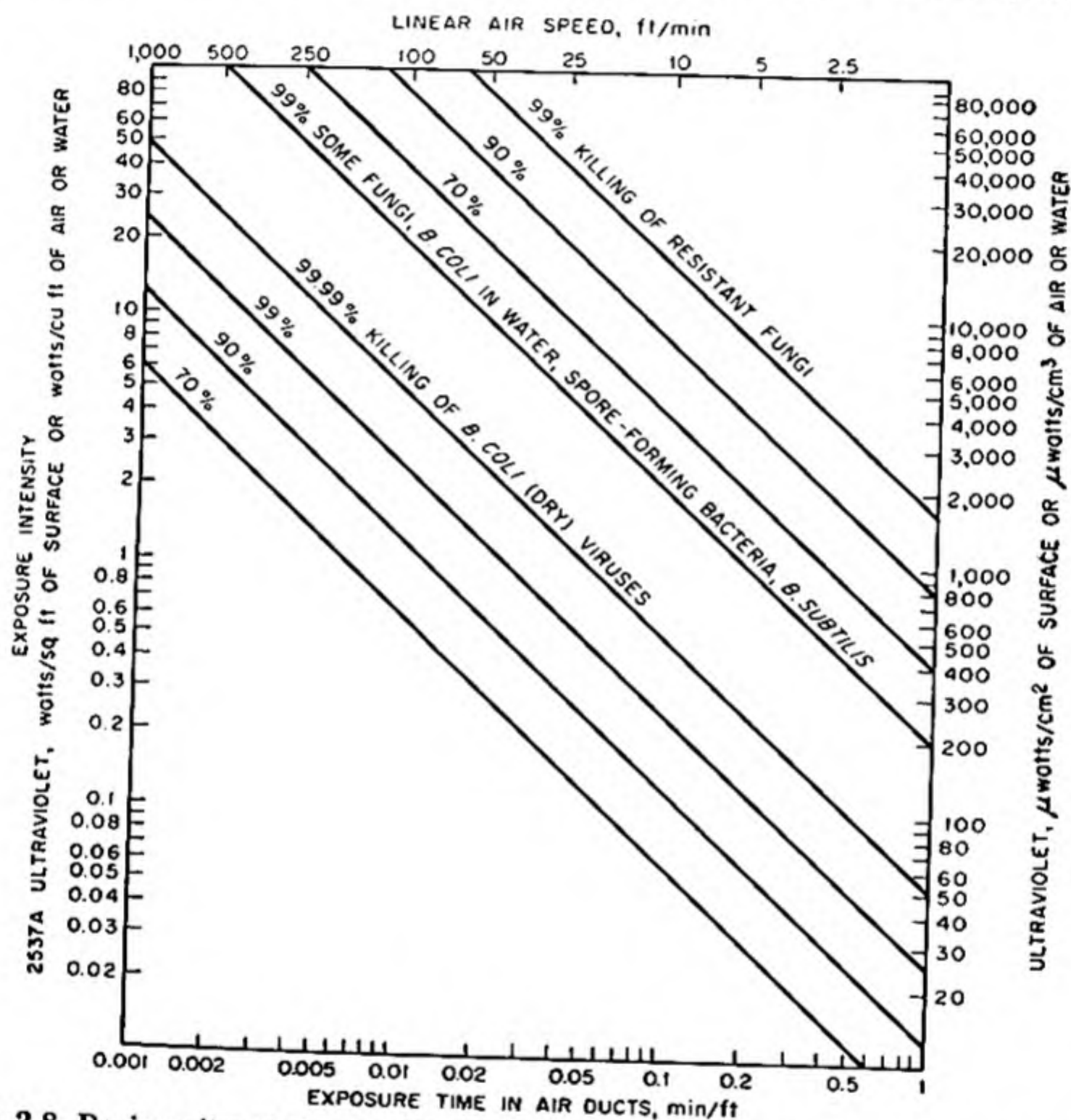


FIG. 2-8. Reciprocity of time and intensity for short-time exposures in air ducts.

2537 A energy of 450,000 ergs or  $750 \mu\text{W-min/cm}^2$  will produce a minimum perceptible erythema of many skins (see Chap. 13, this volume).

#### AMERICAN MEDICAL ASSOCIATION TOLERANCE

The American Medical Association (1948) specifies  $0.5 \mu\text{W/cm}^2$  as the maximum permissible ultraviolet intensity for 7-hr/day exposures, an exposure of 126,000 ergs, or  $210 \mu\text{W-min/cm}^2$ , but they reduce this to  $144 \mu\text{W-min/cm}^2/\text{day}$  (an intensity of  $0.1 \mu\text{W/cm}^2$ ) for continuous exposure and acknowledge that the specification is based on experience with wave lengths 2800–3200 Å rather than wave length 2537 Å.

Although this exposure tolerance might seem to supply an unnecessary factor of safety, it provides for some unusual skin sensitivities bordering on the pathologic. Experience in hospitals and in industrial applications indicates, for example, that some adult face skins are more sensitive to ultraviolet irritation than the face skin of the average infant. The American Medical Association has not had occasion to specify a corresponding suberythema exposure for 2967 Å ultraviolet, but its unit exposure for a minimum perceptible erythema on average untanned skin is 300  $\mu\text{W}\cdot\text{min}/\text{cm}^2$ , as indicated in Fig. 2-7*b*.

It should be noted here that there is no theory or evidence that the erythema produced by 2537 Å energy differs at all from that produced by 2967 Å except that the former is more superficial and transient. Exposures to comparable erythemas which result in skin peeling after 2967 Å produce only scaling after 2537 Å. This difference in effect, shown in the erythema-action curve of Fig. 2-1, may be due entirely to the slightly deeper penetration of 2967 Å into the skin (see Chap. 13, this volume).

Useful germicidal intensities of 2537 Å ultraviolet range from 5 to 6000  $\mu\text{W}/\text{cm}^2$ , and the corresponding suberythema exposure times are from less than 40 min to 2 sec. The intensity of ultraviolet reflected from surfaces and walls of a minimum reflectance of 5–10 per cent may range from 5  $\mu\text{W}$  downward. From this it is obvious that there is always a problem of face and eye protection in practical applications of ultraviolet energy.

Table 2-2 extrapolates the American Medical Association's permissible exposure through practical ranges of time and intensity.

TABLE 2-2. MAXIMUM PERMISSIBLE DAILY EXPOSURES

Exposure time per 24 hr, hr	Intensity on faces, $\mu\text{W}/\text{cm}^2$	Exposure time per 24 hr	Intensity on faces, $\mu\text{W}/\text{cm}^2$
24	0.1 <sup>a</sup>	2 hr	1.8
18	0.2 <sup>a</sup>	1 hr <sup>b</sup>	3.6
12	0.3	30 min	7.2 <sup>c</sup>
9	0.4	10 min	21.6 <sup>c</sup>
6	0.6	1 min <sup>d</sup>	216.0
4	0.9	30 sec	432.0
3	1.2	5 sec	2600.0

<sup>a</sup> Permissible intensity in hospital infant wards; one-fiftieth or one one-hundredth that recommended for hospital air disinfection.

<sup>b</sup> Exposures (time times intensity) of 3.6  $\mu\text{W}\cdot\text{hr}/\text{cm}^2$ .

<sup>c</sup> Intensity recommended for hospital upper-air disinfection; tolerated only 10–30 min if on the faces of personnel.

<sup>d</sup> Exposures (time times intensity) of 216  $\mu\text{W}\cdot\text{min}/\text{cm}^2$ .

FACE AND EYE PROTECTION AND TREATMENT

Commercially available sun glasses and face shields designed to cover the eyes from the sides and the ears completely provide adequate protection. Hands and arms may be protected by plastic or rubber or very



closely woven textile gloves, but these gloves should be tested before long-time use.

The discomfort from ultraviolet-irritated eyes may be relieved by exposing them for 15–20 min to as high an intensity of heat as can comfortably be borne from a heat lamp or from an ordinary 50- to 60-watt incandescent lamp held close to the eyes; the treatment is effective through closed eyelids (author's personal experience). In extreme cases, a doctor should be consulted, but when this is impracticable, the usual first-aid treatment is the application of ice packs. In any case, the irritation produced by the ultraviolet may disappear within a day or two and much more quickly than a corresponding degree of irritation from a longer wave ultraviolet source. A severe conjunctivitis may, however, make the eyes susceptible to secondary infection until the lesions are healed.

### COMMERCIAL SOURCES OF ULTRAVIOLET

Mercury-vapor sources of ultraviolet for practical and experimental uses may be grouped as (1) commercially available low-pressure (0.004–0.02 mm of Hg) germicidal lamps, (2) high-pressure (400–60,000 mm of Hg or 0.5–75 atm) photochemical, therapeutic, and filtered sunlamps, and (3) special experimental lamps of limited availability. The characteristics of all but the low-pressure lamps are discussed later in this chapter.

Arc lamps with rare-earth cored carbons provide powerful sources of energy for many photochemical, photographic, and photocopying applications of the ultraviolet. For various reasons their biological applications have been rather limited and are not discussed in this chapter.

### HIGH- AND LOW-PRESSURE MERCURY ARCS

As indicated graphically in Fig. 2-5, low-pressure mercury arcs are 5–10 times more efficient in germicidal action than high-pressure arcs in envelopes of the same transmission. It should be noted that high-pressure quartz-mercury arcs may be of practical use, regardless of efficiency, in places where it is impossible to provide the essential ultraviolet intensities from the much more bulky low-pressure lamps. For example, the germicidal effect per unit of total volume of a 360-watt high-pressure arc in quartz is 5–10 times that from low-pressure arcs, but the germicidal efficiency of the high-pressure arc is one-fifth to one-tenth that of the low. For another example, the germicidal effectiveness of the radiating part of the UA-3 and UA-11 high-pressure mercury arcs of Table 2-6 can be duplicated only by 8–12 times the radiating length of the more efficient low-pressure lamps.

### ULTRAVIOLET OF WAVE LENGTH 2537 Å

#### INHERENTLY LOW EMISSION INTENSITY OF SOURCES OF 2537 Å

The possibility of a source of 2537 Å ultraviolet with the high power output per unit of source area, or radiant-flux density, of the high-pressure





WL-782-20 <sup>i</sup>	14	2.0	20	24.75	20	0.625	950	325	0.055	4,500
2852Q <sup>i</sup>	14	3.3	36	29.375	24	0.520	3000	450	0.030	12,000
WL-782-30 <sup>i,k</sup>	17	5.2	46	34.75	30	0.625	950	410	0.050	12,000
ST34A18 <sup>i</sup>	18	4.5	50	34.5	30	0.620	2000	375	0.045	12,000
ST34B18 <sup>i</sup>										
ST34I18 <sup>o</sup>										
ST46A22 <sup>i</sup>	22	3.4	34	16.125	11	0.6875	600	200	0.120	12,000
ST30A32 <sup>i</sup>	32	7.0	77	30.125	25	0.6875	600	300	0.120	12,000
ST96S30 <sup>i</sup>	36	8.0	88	34	96	0.620	3000	800	0.045	7,500
G36T6 <sup>e,j</sup>	16-17	6.3-8.0	65-70		29.25	0.625-0.75	450-660	150-180	0.100-0.120	2,500 <sup>h,m</sup>
G36T6 <sup>e,j</sup>	23-27	8.7-12.2	90-105		29.25	0.625-0.75	450-660	135-150	0.200-0.220	2,500 <sup>h,m</sup>
G36T6 <sup>e,j</sup>	30-34	10.6-13.7 <sup>n</sup>	110-120 <sup>n</sup>	34	29.25	0.625-0.75	450-660	115-130	0.300-0.320	2,500 <sup>h,m</sup>
G36T6 <sup>e,j</sup>	36-39	11.6-14.8 <sup>n</sup>	120-130 <sup>n</sup>	34	29.25	0.625-0.75	440-450	105-120	0.420-0.430	2,500 <sup>h,m</sup>

<sup>a</sup> Data for lamps listed by more than one manufacturer represent an industry average which may be slightly different from the specific data on each manufacturer's product.

<sup>b</sup> Initial output may be 25 per cent higher.

<sup>c</sup> Multiply by 0.85 for the bactericidal equivalence of 2650 Å in ultraviolet watts.

<sup>d</sup> Continuous operation.

<sup>e</sup> General Electric Company designation.

<sup>f</sup> Sylvania Electric Products, Inc., designation.

<sup>g</sup> Bent-tube construction makes lamp 1.125 in. wide.

<sup>h</sup> At 3 hr per start.

<sup>i</sup> Hanovia Chemical & Manufacturing Co. designation.

<sup>j</sup> Westinghouse Electric Corporation designation.

<sup>k</sup> WL-782L-30, little or no 1849 Å for ozone production; WL-782H-30, high 1849 Å for ozone production.

<sup>l</sup> Spiral, 12 in. in diameter.

<sup>m</sup> At 3 hr per start. Life is 5000 hr at 6 hr burning per start; 7500 hr at 12 hr burning per start.

<sup>n</sup> In still air at 80°F; the output is increased in cool or moving air.



mercury arcs has long tantalized experimenters. From the well-known absorption of the 2537 Å resonance line by mercury vapor and from an erroneous association of efficient 2537 Å production solely with low mercury-vapor pressure, the experimenters have inferred that water or air cooling of a lamp should permit a great increase in the electric power input and the 2537 Å emission. In the search for the optimum conditions for 2537 Å production, rather definite optima of vapor pressure and power input, corresponding to lamp-tube temperatures of 40°–60°C, have been found. Radical decreases in mercury pressure by cooling or increases in power input, either separately or concurrently, produce relatively small changes in 2537 Å output but produce radical changes in efficiency. The output ratings of commercial sources are the maxima consistent with good efficiency and life. Users of 2537 Å sources who may be willing to sacrifice both life and efficiency for higher output power density must now be reconciled to a maximum emission of the order of 30–50 ultraviolet mw/cm<sup>2</sup> of source surface provided by about 0.1–0.15 watt of electrical power input per square centimeter of tube surface. This is about twice the output of commercial sources. The difference between power input and emission (3 to 1) results from the inefficiency of the conversion of electrical power to radiant power in the lamps and a subsequent absorption of about 20 per cent in the glass tube. Tenfold increases in power input, which are possible by water or air cooling, provide but slight increases in the 2537 Å output per unit of source area. These generalizations have little or no bearing on the radiation characteristics of higher pressure mercury arcs discussed later.

#### SOURCES OF 2537 Å ULTRAVIOLET

Table 2-3, based partially on the IES Lighting Handbook, 2d ed. (1952), presents the physical, electrical, and radiation characteristics of most of the commercially available sources of 2537 Å energy. In each case the amount of electrical input (in watts) to the arc, the length of the radiating source, and the total radiated 2537 Å energy in watts, hereafter called "ultraviolet watts," and the ultraviolet watts per square centimeter at a distance of 1 meter are closely associated. This permits calculation of the efficiency of the sources, of their input and output per unit of source length and area, and of the ultraviolet intensity provided by them at various distances. Division of the intensity in ultraviolet microwatts per square centimeter at 1 meter by 10,000 provides a useful practical rating in ultraviolet watts per square foot at 10 ft. Multiplication of ultraviolet microwatts per square centimeter at 0.9290 converts to ultraviolet milliwatts per square foot, but for practical purposes they are equivalent. Multiplication of the 10-ft rating by 100 and division by the distance squared provides intensity in the same units for other distances greater than the length of the radiating source.

## CONVERSION FACTORS

## POWER, INTENSITY AND DISTANCE, AND WORK AND ENERGY

The use of metric and U.S. units, separately and together, named units, and various time units, with little standardization of practice, requires frequent use of conversion factors. In Table 2-4 are listed various con-

TABLE 2-4. CONVERSION FACTORS

From	Multiply by	To
Power Output and Intensity		
UV watts output, total.....	10.0 <sup>a</sup>	UV $\mu\text{W}/\text{cm}^2$ at 1 meter
UV watts output, total.....	0.001	UV $\mu\text{W}/\text{sq ft}$ at 10 ft
UV $\mu\text{W}/\text{cm}^2$ .....	0.929	UV mW/sq ft
UV output/steradian.....	10.0 <sup>a</sup>	UV output total
UV output/steradian.....	0.01	UV output/cm <sup>2</sup> at 10 cm
UV output/steradian.....	0.0001	UV output/cm <sup>2</sup> at 1 meter
UV intensity, unit area, 1 meter...	0.176	UV intensity, unit area, 10 ft
UV intensity/cm <sup>2</sup> at 1 meter.....	$10 \times 10^3$	UV intensity/sq ft at 10 ft
ergs/sec.....	0.1	UV $\mu\text{W}$
joules/sec.....	1.0	UV watts
Work and Energy <sup>b</sup>		
joules.....	1.0	UV watt-sec
joules.....	$0.01665 \times 10^6$	UV $\mu\text{W-min}$
ergs.....	0.1	UV $\mu\text{W-sec}$
ergs.....	0.011665	UV $\mu\text{W-min}$
g-cal.....	$69.77 \times 10^3$	UV $\mu\text{W-min}$
Btu.....	$17.4 \times 10^6$	UV $\mu\text{W-min}$
r (roentgens) in air.....	0.00018315	UV $\mu\text{W-min}$
Photons or quanta at 2536 A.....	$0.0131 \times 10^{-12}$	UV $\mu\text{W-min}$
Exposures		
joules/cm <sup>2</sup> .....	$0.01665 \times 10^6$	UV $\mu\text{W-min}/\text{cm}^2$
ergs/cm <sup>2</sup> .....	0.001665	UV $\mu\text{W-min}/\text{cm}^2$
g-cal/cm <sup>2</sup> .....	$69.77 \times 10^3$	UV $\mu\text{W-min}/\text{cm}^2$
r/cm <sup>2</sup> .....	0.00018315	UV $\mu\text{W-min}/\text{cm}^2$

<sup>a</sup> Approximate.

<sup>b</sup> When on equal areas—exposures.

versions to the centimeter-microwatt-minute units used in this chapter. The relation between ultraviolet output and maximum intensity refers only to essentially linear sources, to distances greater than the length of the source, and to directions of maximum intensity perpendicular to the center of the source. Energy per steradian refers only to the steradian in that maximum intensity direction and, literally, only to a very small part of the solid angle represented by the steradian. For this reason, energy



per steradian is an unsatisfactory description of the output of linear sources.

### 2650 Å EQUIVALENCE

Since 2537 Å energy has only about 85 per cent of the bactericidal action of 2650 Å energy (Table 2-1), the ultraviolet watts output and ultraviolet microwatts of Table 2-3 must be multiplied by 0.85 for the 2650 Å bactericidally equivalent ultraviolet watts of Table 2-6.

## INTENSITY

### RELATIVE ENERGY DISTRIBUTION

The inherent spectra of all low-pressure mercury arcs are dominated by the 2537 and 1849 Å lines. Other lines are so relatively weak and from such low-intensity sources that they are of little practical value (Table 2-5 and Fig. 2-5b). The output of the 1849 Å line is determined

TABLE 2-5. RELATIVE ENERGY IN VARIOUS SPECTRAL LINES OR GROUPS OF TYPICAL LOW-PRESSURE LAMPS

Wave Length, Å	Relative Energy, %
2537	100
2652	0.14
2753-2893	0.12
2967	0.37
3022	0.17
3126-3132	1.43
3650-3663	1.30
3906-4077	1.60
4339-4358	3.40
5461	2.25
5770-5791	0.60

over a wide range by the fused quartz and the special glasses, in various thicknesses, whose transmissions are shown in Fig. 2-5a. Since the 1849 Å energy is rapidly absorbed by air (about 50 per cent in 1 in.) and since it penetrates liquids and cellular proteins much less effectively than the 2537 Å, little practical bactericidal application has been found for energy of this wave length. Since 1849 Å energy is only slightly absorbed by nitrogen but is readily absorbed by oxygen, it provides ozone in air, relatively uncontaminated by oxides of nitrogen, and some practical application of this energy for this purpose is being made. Commercially available sources in thin glass provide 1849 Å energy to an extent 1-2 per cent that of 2537 Å energy. Greater 1849 Å energy output is possible through thin fused-quartz glass.

### RESEARCH SPECIFICATIONS OF ULTRAVIOLET INTENSITY

The ultraviolet power output and intensity ratings of Table 2-3 are average values for new sources. The variation and service depreciation

of all commercial sources are such that the output ratings should be used for approximations only. A description of a lamp type and of its electrical characteristics is an essential guide to the mechanics of a laboratory research but is of little value as a means of specifying the radiation intensities provided. The effective intensities obtained in any research should be measured at the irradiated surface or throughout the irradiated volume and should be specified in general terms entirely independent of the source, the usual laboratory unit being the microwatt per square centimeter, and the corresponding engineering unit, the milliwatt per square foot.

#### INTENSITY VARIATIONS WITH DISTANCE

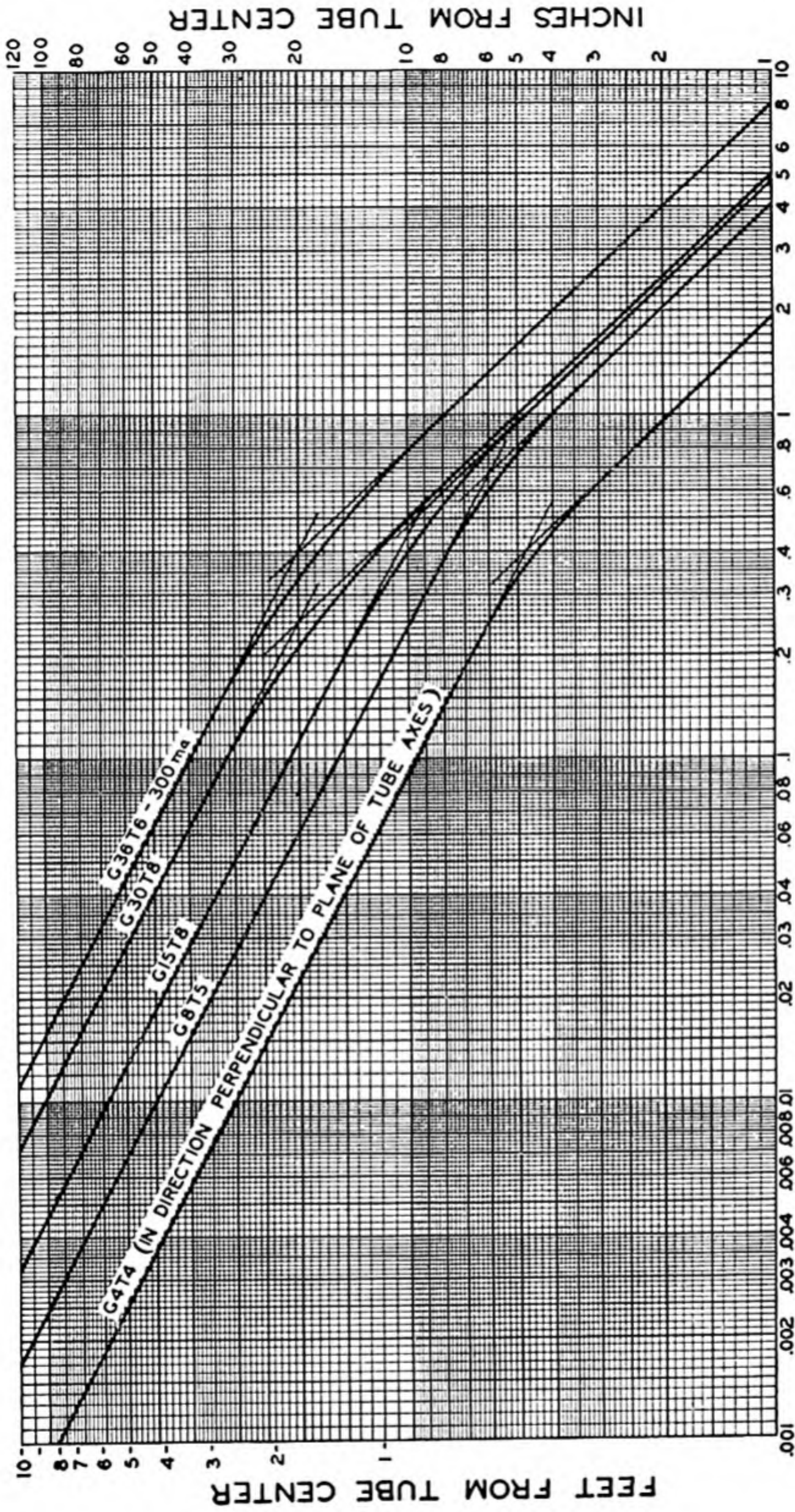
For distances greater than the length of the radiating source, the intensity varies inversely as the square of the distance. For distances less than about one-third the radiating length of these linear type sources, the intensity varies inversely as the distance. The variation at the transition distances can be measured directly or estimated from the actual intensities produced by such typical sources as are shown in Fig. 2-9.

#### HIGH INTENSITIES AT CLOSE RANGE

The maximum intensity provided by a single tubular source is at its own surface. This is a useful point at which to start a study of the variation of intensity at short distances from the tube. Distances are measured from the center of the tube although it radiates ultraviolet as if only the surface of the tube were the source. The effective emitting length of the 1-in. G30T8 tube of Fig. 2-9 is 32 in., and its circumference is 3.14 in. From this surface area of about 100 sq in., 7 ultraviolet watts is radiated, an emission intensity of 0.07 ultraviolet watt/sq in. or  $10,800 \mu\text{w}/\text{cm}^2$ . A surface or a material in contact with the tube would therefore be irradiated at that intensity.

Similarly, a cylindrical surface 2 in. in diameter would have double the tube area. It would intercept practically all the radiated 7 ultraviolet watts with half the intensity. Similarly, the intensity on a 3-in. cylindrical surface would be one-third as great, and on a 4-in. cylinder, one-fourth as great. On cylinders of these diameters, small compared with their length, the ultraviolet intensity is uniformly distributed, except for a length at each end equal to about the radius of the tube. The intensity on the surfaces of such irradiated cylinders varies with their diameters, or inversely as the distance from the tube axis to the irradiated surface. This relation is true out to distances of the order of one-third the effective length of the source, as shown in Fig. 2-9.





RATED (100 HOURS) ULTRAVIOLET WATTS PER SQ. FT.

FIG. 2-9. Variation of intensity with distance from typical bactericidal tubes. 1 ultraviolet watt = 1000 milli(m)watts = 1,000,000 micro( $\mu$ )watts.



## AVERAGE INTENSITY IN SPACE

In the disinfection of fluids, the average ultraviolet intensity, or average radiant-energy density, throughout the concentric cylindrical space surrounding the tubular sources becomes a basic factor. It is especially basic in the case of air where there is no absorption to modify the linear decrease in intensity inversely with the distance from the lamp tube. It can be the basic intensity factor in the disinfecting exposure of air where it is possible to provide enough turbulence of flow to expose the air to the full range of intensities, and so to an average intensity, during its travel through an irradiated zone. Since the intensity at less than source-length distances from linear sources varies inversely as the distance and the volumes of successive increments of annular space increase directly as the distance, the products of annular volume increments and their energy density become constant. Thus, within source-length distances, the average intensity occurs at the average distance of one-half the radius of the irradiated zone and is twice the intensity at the outer limits of the zone, as pointed out by Luckiesh and Holladay (1942a,b).

At distances greater than source length, as in directly irradiated rooms or very large plenum chambers of ventilating systems, the intensity throughout the spherical space surrounding a central ultraviolet source varies inversely as the square of the distance, and the volumes of successive increment shells of space increase directly as the square of the distance, so that the products of successive volume increments and their energy density become constant. Here again the average intensity would occur at the average distance of  $1/\sqrt{3}$  or 0.577 the radius of the irradiated spherical volume and would be three times the intensity at the outer limits of the volume, as developed by Wells (1940), if it were not for the toroidal rather than spherical spatial distribution of the energy about a linear source. Also, since irradiated rooms are cubical rather than spherical in form, the average intensity occurs at more nearly half the average radial distances to the walls and is again about half the intensity at the outer limits of the spherical or cubical zone.

## INCREASE OF INTENSITY AND UTILIZATION BY REFLECTORS

Efficient sources of the germicidal ultraviolet are inherently low in intensity compared with high-pressure sources designed for photochemical and therapeutic use. A maximum intensity of 10–20 ultraviolet watts/sq ft (10,000–20,000 ultraviolet- $\mu\text{w}/\text{cm}^2$ ) can be available at a tube surface for experimental work, but, at practical working distances, only about one-fourth this intensity can be obtained as irradiation over an extended area (see Fig. 2-10b).

The ionized mercury vapor in germicidal tubes almost completely absorbs any 2537 Å energy which might otherwise pass through the glass tube itself from an outside source. For this reason, only the thin layer



of mercury vapor practically in contact with the lamp tube is an effective source of 2537 Å energy. Thus, when several such tubes are placed in contact, side by side to form a grid, only about one-third their total ultraviolet output reaches a parallel irradiated surface of an area about one-third the total surface areas of the tubes (Fig. 2-10a). It is of interest to note that, with such a rectangular or square grid source formed by such an assembly, the intensity on the irradiated surface remains constant for distances out to about half the width of the assembly. As the distances are increased to the length of the rectangle, there is a transition to a variation inversely with the square of the distance. Single tubes are, in effect, rectangles of so small a width as to have the characteristics of theoretical line sources (Fig. 2-9).

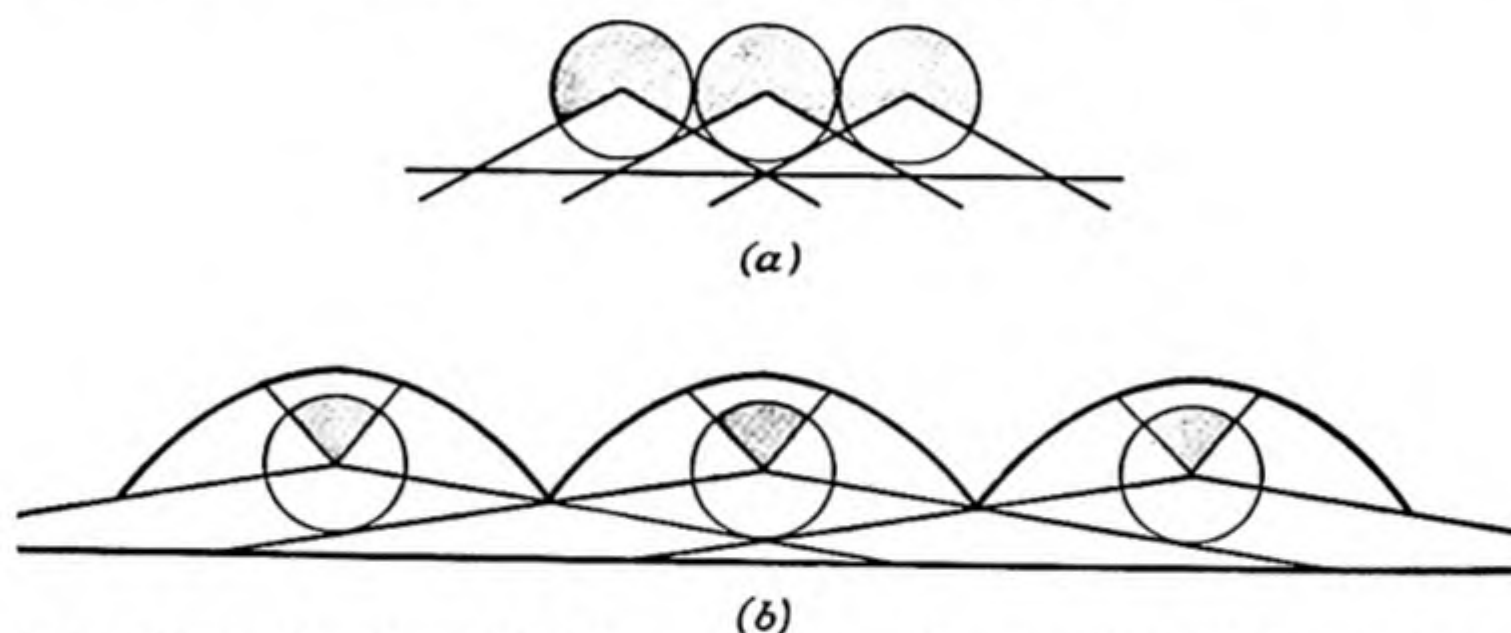


FIG. 2-10. Effect of bactericidal-tube spacing on utilization of total ultraviolet output. (a) Close spacing. (b) Open spacing under reflectors.

The high intensities possible with germicidal tubes in contact in a grid pattern can be produced more economically with about one-third as many tubes fitted with reflectors. In a tube-and-reflector system the tubes should be spaced on centers three or four times their diameter (Fig. 2-10b).

All reflectors for practical uses with germicidal lamps should be of specially processed, polished aluminum (60–70 per cent reflectance) or polished chromium plate (40–50 per cent reflectance). Luckiesh and Taylor (1946) have shown that no other reflecting materials are of practical value. Special aluminum paint may be used in some places, such as in air ducts, if the service or maintenance is such as to make occasional repainting practical. Such a paint is made of pure aluminum flakes in a vehicle of plastic lacquer of high ultraviolet transmission.

Specular aluminum reflectors, designed to intercept about two-thirds of the tube energy, redirect about 65 per cent of the energy to the irradiated surface. The efficiency is therefore 65 per cent of two-thirds or 43 per cent plus the 33 per cent directly from the tube or a theoretical total of  $\sim 75$  per cent. In practice, commercial equipment is only capable of doubling the effective radiation from an equivalent grid of bare tubes.

The mercury column in the tube absorbs almost all the energy redirected to it by the reflector and prevents higher utilization.

### OZONE FORMATION

One-tenth to 5 per cent of the mercury line 1849 Å energy is transmitted by the glasses used for the tubes of germicidal lamps as indicated in Fig. 2-5a. Energy of this wave length, transmitted only a few inches through air, easily breaks the weak bonds of the oxygen molecule to permit the formation of ozone near the ultraviolet source. Koller (1946) found such ozone to have a half life of 15 hr, from concentrations of several hundred parts per million, in the dry glass containers in which it had been formed. Ewell (1942) had found, however under more practical conditions, that humidity, light, 2537 Å ultraviolet, and surface absorptions greatly catalyzed the reversion to oxygen. Under such conditions he found ozone to have a half life of 2-3 min when irradiated with 2537 Å ultraviolet and of 6-7 min when not irradiated, in both cases from concentrations of 3-4 ppm.

Since this ozone diffuses throughout an irradiated space, its inherent instability is considerably increased by 2537 Å energy, and short-lived atomic oxygen occurs in a unique manner. Commercial sources permit air disinfection with equilibrium ozone concentrations less than the 1 part per 10 million considered permissible by the American Medical Association (1948). Other sources provide the somewhat higher concentrations traditionally used in certain food-storage applications, where the odor-masking effects of ozone and its concentration by absorption on moist surfaces may be of some value.

### PHOTOCHEMICAL EFFECTS OF 2537 AND 1849 Å ENERGY

The photochemical actions of the 2537 and the 1849 Å energy are outside the scope of this chapter except in so far as they are incidental to some of the practical applications in other fields. The 2537 Å energy considerably increases the normal oxidizing action of oxygen without its obvious ionization. This is greatly increased wherever there is ionization and ozone formation by 1849 Å energy. In all germicidal applications of the ultraviolet the possibility of objectionable chemical changes should be investigated, e.g., formation of phosgene and hydrogen chloride in poorly ventilated dry cleaning rooms where carbon tetrachloride may be used, formation of hydrogen sulfide and mercaptans in egg-drying plants where egg powder may be in the air, and modification of the flavor of irradiated foods such as meat, milk, cheese, and butter.

### TEMPERATURE AND VENTILATION

Like fluorescent lamps, commercial germicidal lamps are designed to operate under average conditions of room temperature and ventilation.



Unusual enclosure or extremes of air temperature, such as in refrigerators, ovens, and air ducts, will reduce the ultraviolet output of the germicidal tube to the same extent as the light output of a similar fluorescent lamp. The reduction is about 10 per cent at 50° and 100°F, 20 per cent at 40° and 110°F, and 30 per cent at 35° and 120°F.

### DEPRECIATION

In common with fluorescent lamps, bactericidal tubes depreciate rapidly during the first 100 hr of operation. This is considered a part of the manufacturing process, and commercial lamps are given an initial rating as if at 100 hr of normal operation. In Fig. 2-11 the approximate

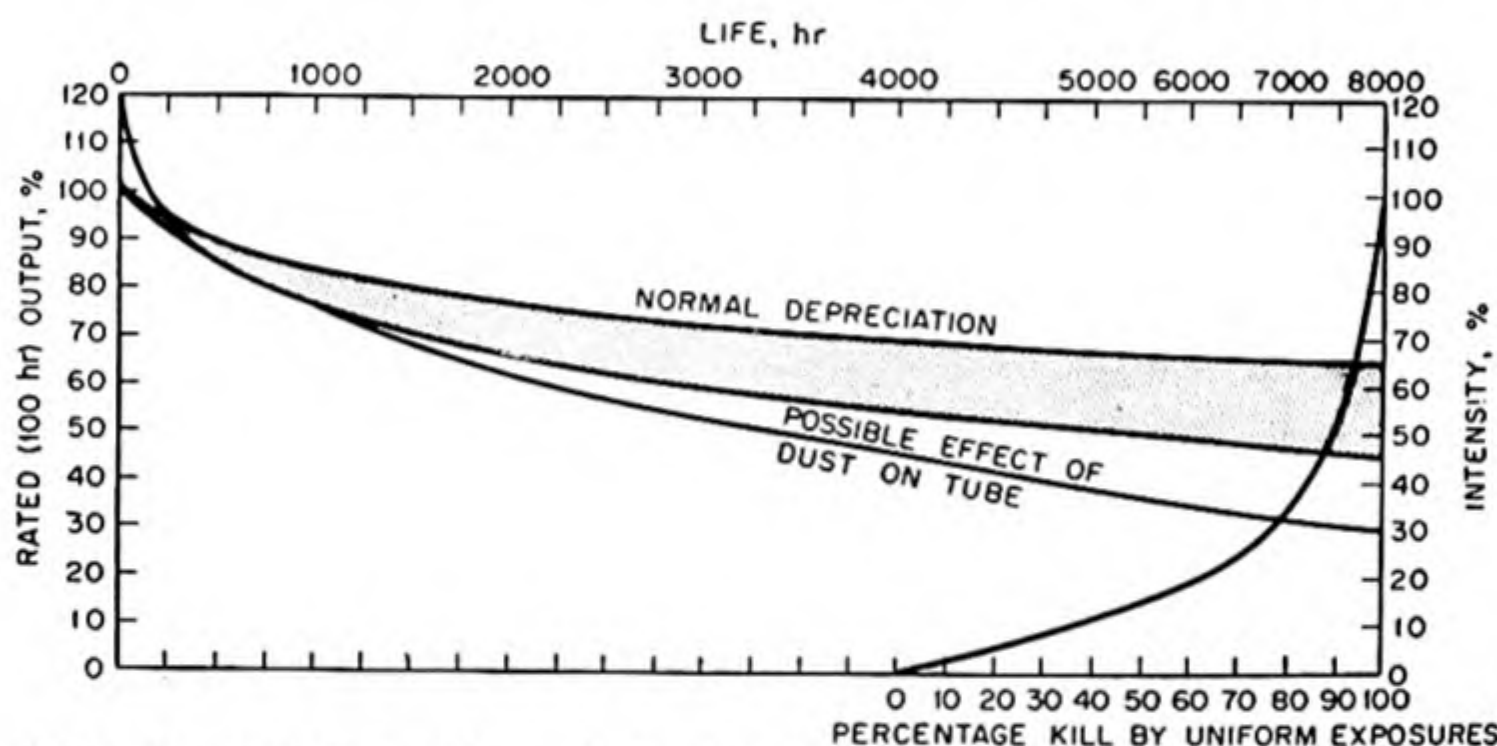


FIG. 2-11. Depreciation, life, and bactericidal-effectiveness curves of typical low-pressure ultraviolet sources.

depreciation of low-pressure sources is shown. Those in high-transmission glasses start from 20 to 25 per cent above the 100-hr rating, whereas those in fused-quartz glass or in similar Vycor glass start from only slightly above the 100-hr rating, considered as 100 per cent in Fig. 2-11.

The depreciation rate, and therefore the effective life, is affected by the length of the operating periods; the shorter the average operation per start, the faster the depreciation and the shorter the effective life. In general, such lamps fail to start and operate normally at about the end of their effective life, i.e., at about 60 per cent of their 100-hr rating. Lamps with so-called "cold" electrodes provide some exception to these rules, but they still have the basic depreciation limitations of constant operation. They may continue to start and operate normally after their output has dropped to ineffective levels.

It should be noted that ultraviolet killing is an exponential rather than linear function of ultraviolet intensity. The curve at the right of Fig. 2-11 provides a typical illustration of the relatively small amount of change in ultraviolet killing that may result from a large depreciation.

## ULTRAVIOLET DISINFECTION

Practical uses of the lethal action of ultraviolet energy are limited only by the practicality, in each case, of providing an adequate exposure (intensity times time) on microorganisms. Exposure time is usually defined by mechanical conditions leaving little chance for radical change, and the exposure intensity must be adjusted for an adequate exposure product. Provision of an adequate ultraviolet intensity is conditioned on two basic and independent factors: (1) the variation with distance from the source or sources and (2) the absorption of the ultraviolet by intervening media. The variation with distance has been represented in general terms as insignificant within a few inches from a relatively large assembly of sources and reflectors, as inverse with the distance for a few inches from single tubular sources and reflectors and a few feet from large-area sources, and as inverse with the square of the distance at greater than source-length distances from single tubular sources and reflectors, as well as at considerably greater distances from large areas. The absorption of germicidal ultraviolet by air is entirely negligible even for the irradiation distances of large auditoriums.

## AIR DISINFECTION

Ultraviolet air disinfection is commonly accomplished by placing germicidal lamps in the rooms or in the air ducts serving such rooms. The two methods well illustrate the definition of exposure times by the mechanics of the problems, with adequate exposure intensities to be provided if possible. As detailed later, an effective exposure for air disinfection is 15,000 ergs/cm<sup>2</sup> or 25 ultraviolet  $\mu$ w-min/cm<sup>2</sup>. In the upper air of occupied rooms, practical exposure times may be 1-5 min, and effective intensities may be 25-50 ultraviolet  $\mu$ w/cm<sup>2</sup>. In air ducts the exposure times may be  $\frac{1}{8}$ - $\frac{1}{2}$  sec, and the corresponding intensities may be 10,000-25,000 ultraviolet  $\mu$ w/cm<sup>2</sup>.

## DUCT AIR DISINFECTION

The disinfecting exposure of duct air is defined by its transit time through an ultraviolet-filled zone of definite length and by the average ultraviolet intensity during the transit time throughout that zone. In Fig. 2-2, 15 ultraviolet  $\mu$ w-min/cm<sup>2</sup> is suggested as a disinfecting exposure for most air-borne microorganisms except fungi. In an air duct with a cross section of 2 sq ft and a rating of 1200 cu ft/min, the linear flow of 600 ft/min through an ultraviolet-filled zone 3 ft long provides an exposure time of 0.005 min. The average ultraviolet intensity throughout the zone must then be 3000 ultraviolet  $\mu$ w/cm<sup>2</sup>, or about 1800 ultraviolet mw/sq ft for an exposure of 15 ultraviolet  $\mu$ w-min/cm<sup>2</sup>. The average intensity throughout a cylindrical zone radially irradiated by a linear



source on its axis of a length greater than the diameter of the zone has been shown to be that on a concentric cylindrical surface of one-half the radius. It follows then that the ultraviolet intensity at any given distance from a germicidal lamp, as indicated in Fig. 2-9, will be the average intensity throughout a cylindrical zone of a radius twice that distance. Thus the G36T6 lamp of Fig. 2-9 provides an intensity of 2800 mw/sq ft at a distance of  $2\frac{3}{4}$  in. and so an average of that intensity throughout a cylindrical space of about the length of the ultraviolet source and a radius of  $5\frac{1}{2}$  in., or a cross-section of 0.95 sq ft. Although two such tubes would provide an effective initial average intensity in the cross section of the duct, three would be specified to increase the minimum intensity at remote parts of the duct and for an effective intensity at the end of tube life. Luckiesh and Holladay (1942a) have developed the theory of ultraviolet duct-air disinfection in minute detail, and Buttolph (1945, 1951) has given it practical application.

*Turbulent Flow for Average Exposure.* In small ducts that require only one or two germicidal tubes which are of necessity placed parallel with the direction of air flow, there may be a 10-to-1 variation in the ultraviolet intensity at distances 1-10 in. from the tube. To ensure that all the air receives an average intensity exposure in its travel through the irradiated zone, either the streamlined flow of the air must be broken into turbulent flow by baffles or more germicidal tubes must be used. In the latter case, as in all cases where many tubes are used, they may be spaced to provide a sufficiently uniform ultraviolet intensity to take care of the streamlined air flow.

*Increase of Average Intensity and Uniformity by Reflective Duct Walls.* Duct walls of pure aluminum of 65-75 per cent reflectance for 2537 Å will nearly double the effectiveness of the germicidal tubes by at once nearly doubling the average intensity and by greatly increasing the uniformity of distribution by multiple reflection.

*Disinfected Duct Air as Alternative to Make-up Air in Sanitary Ventilation.* The most that ultraviolet disinfection can do is to make all the air handled by a duct bacteriologically equivalent to make-up air. Whenever the use of enough ultraviolet to provide a theoretical 99 per cent disinfection of the duct air may be impractical, it should be noted that one-half as much ultraviolet will still provide 90 per cent disinfection, and one-fourth as much will provide about 70 per cent disinfection. In such cases, disinfection provides the equivalent of 90 and 70 per cent make-up air in contrast with the 10-20 per cent usually believed to be economically practical in the winter.

Outdoor air is usually considered satisfactory for the sanitary ventilation of living and assembly quarters, and its usefulness is limited only by the considerable cost of heating and circulating it in adequate quantities. In food and pharmaceutical plants it may, however, carry enough mold

and bacterial contaminants to be a continuous hazard to the products. In these instances, ultraviolet air disinfection can perform a job which is not yet possible by available methods of air washing and filtration.

#### ROOM AIR DISINFECTION

Ultraviolet disinfection of air is accomplished in occupied rooms by germicidal tubes in cylindrical parabolic reflectors which are designed to project the energy for maximum distances through the air of the room above the head level of the occupants. It is accomplished in vacated rooms or where protection of the occupants can be provided by bare germicidal tubes centrally placed in the rooms or on the ceilings. Because of the greater distances the variations in ultraviolet intensities are much greater through irradiated rooms than in irradiated air ducts. Fortunately the convective circulation and the relatively low-intensity long-time exposures practical in irradiated rooms provide an average intensity exposure such as is obtained in air ducts only by induced turbulent flow or by the use of many separate sources of ultraviolet.

*Unoccupied Rooms or Rooms with Occupants Protected.* In the relatively simple case of the vacated room or where occupants may be adequately protected, effective ultraviolet intensities may be provided by centrally placed bare ultraviolet sources. The effective intensity is then determined entirely by the time available to disinfect the air. Assuming this time to be 5 min, the intensity for an exposure of ultraviolet  $\mu\text{w-min/cm}^2$  needs to be only 5 ultraviolet  $\mu\text{w/cm}^2$ . This intensity which can be provided by the G36T6 tube of Fig. 2-9 at a distance of 12 ft will disinfect not only the air but also the walls. Bacteria which might be deposited on the walls from the air are thus subjected to only the minimum intensity present in a 20- by 20-ft room, such as a hospital room between occupancies or a room in a pharmaceutical factory. In rooms where only air-borne bacteria are a problem the average rather than the minimum intensity becomes the basis of installation, and the same bare, centrally placed tube will provide an average intensity of 5 mw/sq ft throughout a 40- by 40-ft room to disinfect the air in only 5 min.

*Occupied Rooms.* Occupants in a room add three serious complications to ultraviolet air disinfection that compel entirely different approaches to the theory and the practice. In the occupied room the problem is not the simple one of cleaning up the residual contamination but the dynamic one of killing or removing air-borne microorganisms as rapidly as they appear from the noses, throats, and clothing of the occupants. The killing or removal must be in such a way as to reduce to a minimum their air-borne life under an equilibrium condition of origin, of necessity several feet from the place of their killing or removal. In the occupied room the maximum intensities tolerated on more sensitive faces range from 0.1 ultraviolet mw/sq ft for continuous exposure to 0.5 ultraviolet mw/sq ft



for 7 hr of exposure per day. Although ideally placed, these intensities are not high enough for rapid disinfection. In occupied rooms the value of air disinfection is primarily that of the health value in removal of microorganisms. In the absence of any criteria of the health value or hazard of air-borne organisms, the natural ventilation believed of value becomes a secondary criterion.

*Convective Circulation and Upper-air Irradiation.* The convective circulation of air, by which the heating of a room from a few localized sources of heat is possible, involves the use of vertical components which provide an interchange of air between the upper and lower parts of a room equivalent to from several air changes per hour to several per minute. In occupied rooms the basic convective circulation is increased by the body heat, the breathing, and the movement of the occupants. These factors increase the circulation in proportion to the crowding, the contamination, and the need for air disinfection. Ultraviolet disinfection of the upper third or fourth of a room can provide in these portions of the room a reservoir of air for the dilution of the lower air at rates equivalent to unusual natural or mechanical ventilation. Lacking more direct criteria of value, it becomes convenient to consider ultraviolet air disinfection as equivalent to and a substitute for outdoor air for sanitary ventilation purposes.

*Upper-air Method of Disinfection.* Luckiesh and Holladay (1942b) treat the upper part of a room as a duct containing air in random circulation at a velocity (5–10 ft/min) equivalent to about one one-hundredth the linear velocity in wall ducts and room units and irradiated with an average ultraviolet intensity (0.025 ultraviolet watt/sq ft) which is about one one-hundredth that provided in air ducts. The upper part of the room is then treated as a duct serving the lower part. There is, however, no such definite separation between the two parts of the room as this oversimplification suggests, and the following analysis (Buttolph, 1951) is believed to be more realistic.

An ultraviolet (2537 Å) intensity of 5 ultraviolet mw/sq ft, effective throughout a cubic foot, will kill respiratory and *E. coli* test organisms at the same rate as they might otherwise be washed or diluted out of the same cubic foot of air by one air change per minute. This is the theoretical reduction of 62.3 per cent of Fig. 2-2. An additional air change or an additional 5 ultraviolet mw/sq ft can dispose of 62.3 per cent of the remaining 37.7 for a theoretical reduction of 82.5 per cent. The effect is the same whether by 5 mw for 2 min or 10 mw for 1 min, two air changes in successive minutes or two air changes in 1 min. These relations are plotted on linear and on semilogarithmic scales for comparison in Fig. 2-12.

An average ultraviolet intensity of 5 mw/sq ft throughout the entire cubage of a room would theoretically provide the disinfection equivalent

in the room of one air change per minute, such as is thought to be desirable in very crowded rooms. Only one-tenth that intensity (0.5 ultraviolet mw/sq ft) is, however, tolerated on sensitive faces for 7 hr per day without objectionable "sunburning." So only about one-tenth of an air change equivalent per minute is practical by this method. This is, however, equivalent to the six air changes per hour, or 30 cu ft of outdoor air per child per minute sometimes specified for school rooms. An average intensity of 5 ultraviolet mw/sq ft throughout a whole room, with only 0.5 permitted in the lower part of the room, can be obtained by an average of 14 mw/sq ft in an upper third of the room or 18.5 in an upper fourth. Since the ultraviolet in the lower part of the room is due mostly to the diffuse reflection from the upper side walls and ceiling, its intensity may vary little throughout the entire area, especially under 10- to 12-ft ceilings. However, since the ultraviolet in the upper part of the room is projected through it from a few sources (usually in cylindrical parabolic reflectors on the walls), the intensity may vary from 2 or 3 ultraviolet mw/sq ft to 2 or 3 ultraviolet watts/sq ft (a thousandfold variation). Such an uneven distribution of the energy in the room is effective only because the convective circulation exposes nearly all the air to nearly the entire range of intensities to provide an integrated lethal exposure, as with turbulent flow in an air duct.

**Hospital Room Disinfection.** The first uses of ultraviolet for air disinfection in hospitals were to provide the equivalent of local curtains or barriers between the surgeon and his operation (Hart, 1936; Overholt and Betts, 1940) and across the front of infant cubicles (Sauer *et al.*, 1942; Del Mundo and McKhann, 1941; Robertson *et al.*, 1939, 1943). Ultraviolet intensities ranging from 20 mw at the floor to 200 mw at head level can readily be provided. The 20-mw intensity becomes as effective as the 200-mw intensity because the width of the divergent beam and the distance of the bacterial travel through the beam, and thus the average exposure time, are ten times as great at the floor. These intensities may produce erythema at head level in 1 min and at floor level in 10 min, so that such installations are limited to pharmaceutical plants

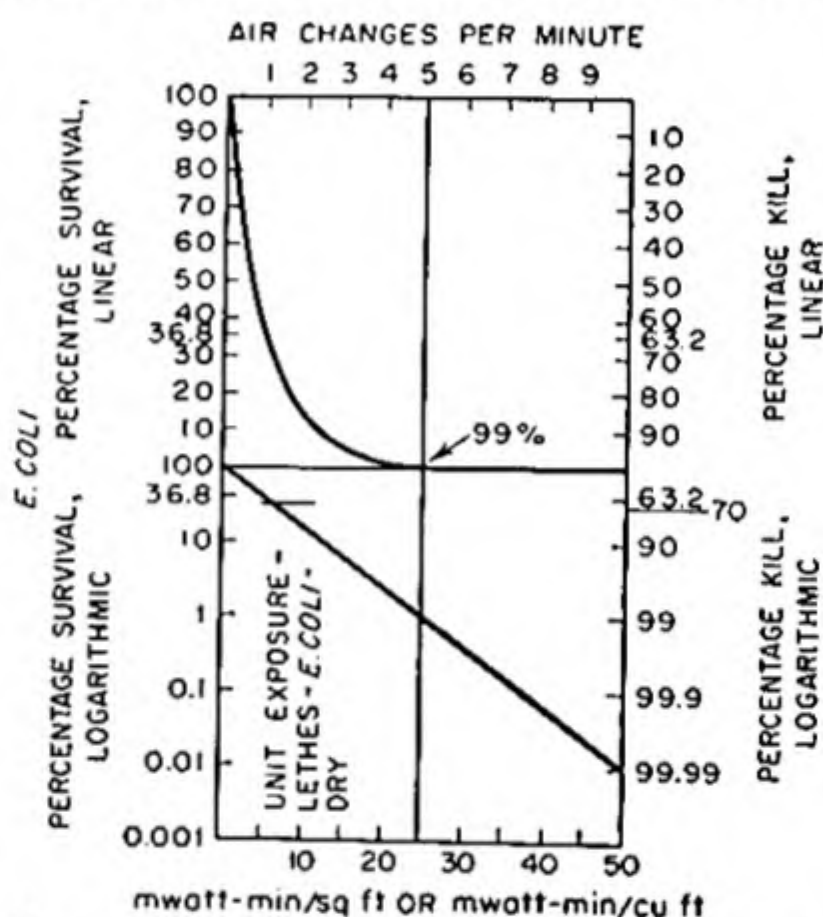


FIG. 2-12. Comparison of linear and logarithmic plotting of percentage survival of air-borne bacteria as a function of dilution by air or by equivalent ultraviolet irradiation.



and infant wards of hospitals where essential discipline of personnel is possible. Such an ultraviolet barrier obviously also provides general air disinfection by virtue of the circulation of room air through it. Because of its positive functioning, the ultraviolet barrier has somewhat the same psychologic as well as engineering appeal as ultraviolet air disinfection in

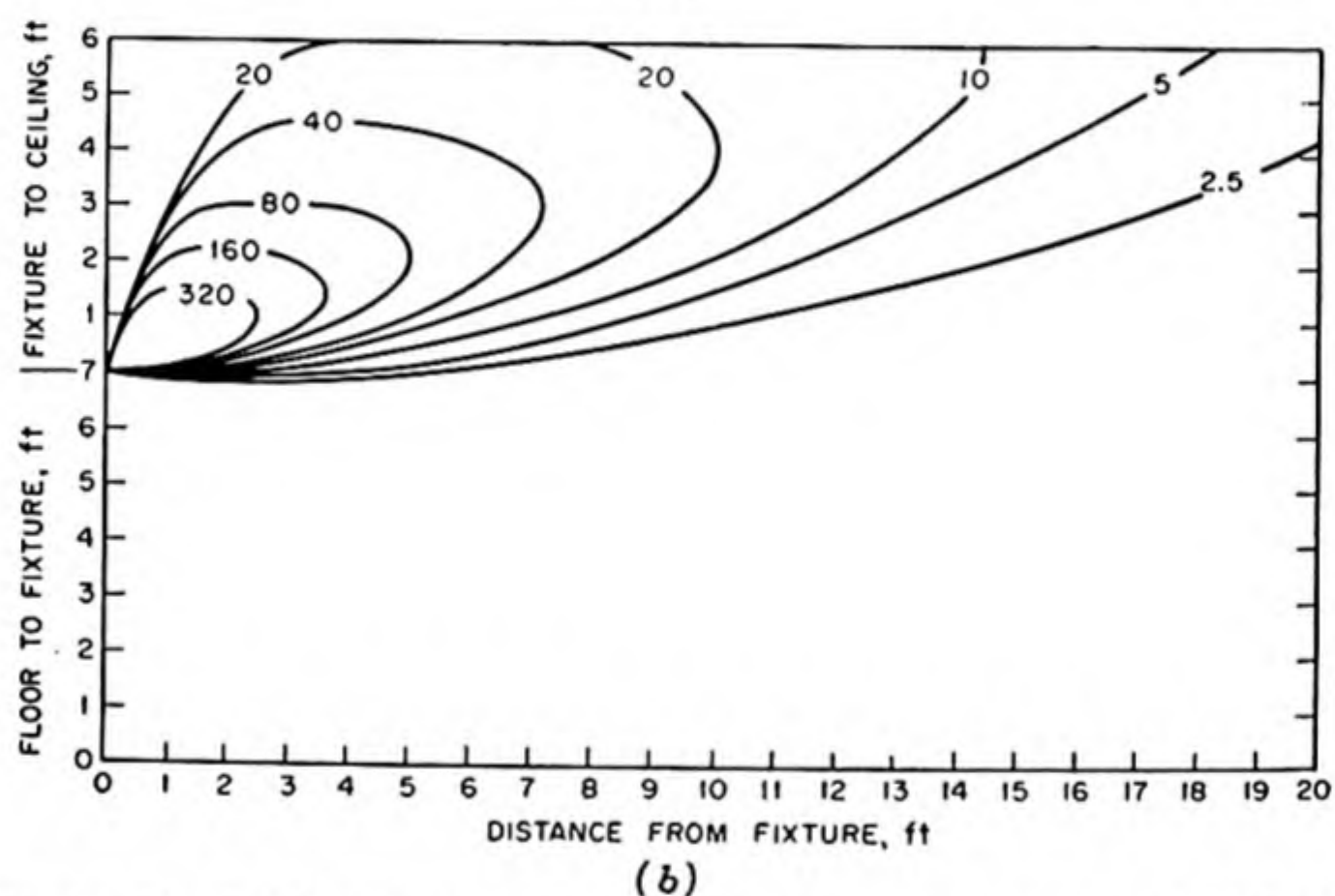
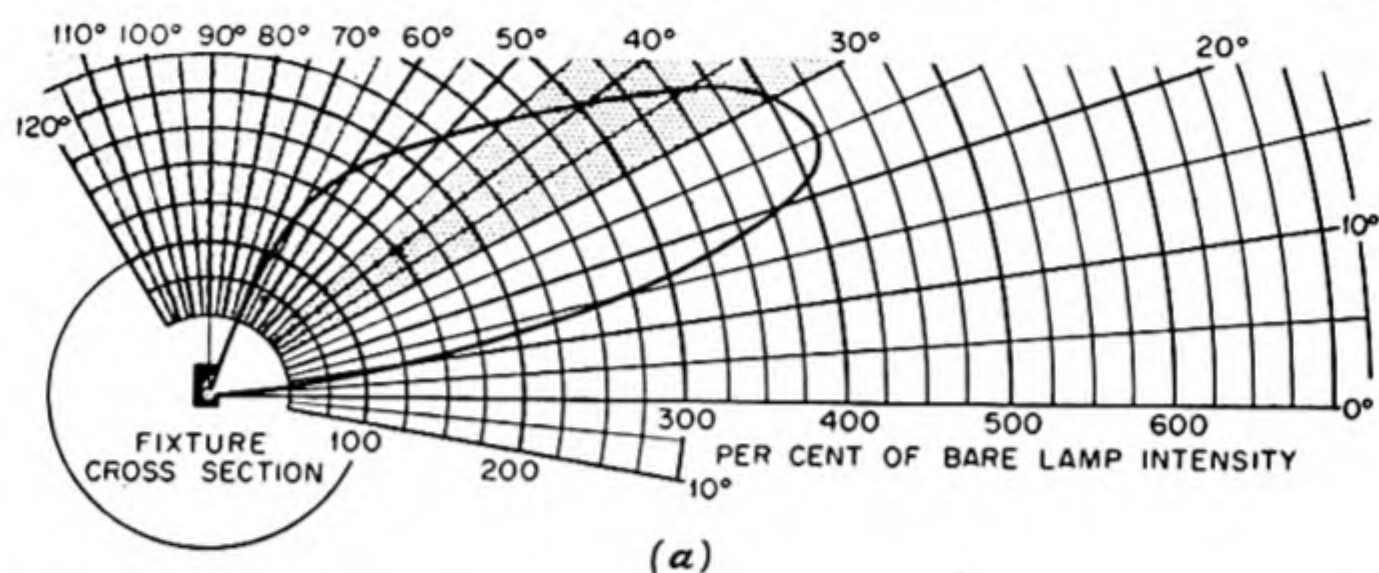


FIG. 2-13. (a) Spatial distribution of ultraviolet from typical bactericidal tubes and open reflectors. (b) Isointensity lines in milliwatts per square foot in a plane perpendicular to the center of the G30T8 tube of Fig. 2-9.

air ducts; the door opening becomes a duct from one room to another in spite of its unconventional cross section compared with its length.

The variation in ceiling height and the difference in exposures per day in patient and service rooms of hospitals have led to two distinct types of commercial equipment, an open type for use under high ceilings and where there may be personnel exposure of about 8 hr per day (Fig. 2-13a) and a louvered type for use under low ceilings and where there may be continuous exposure (Fig. 2-14a). Figures 2-13a and 14b suggest a possible way to provide energy intensities of 15–20 mw/sq ft in the upper third or fourth of a room.

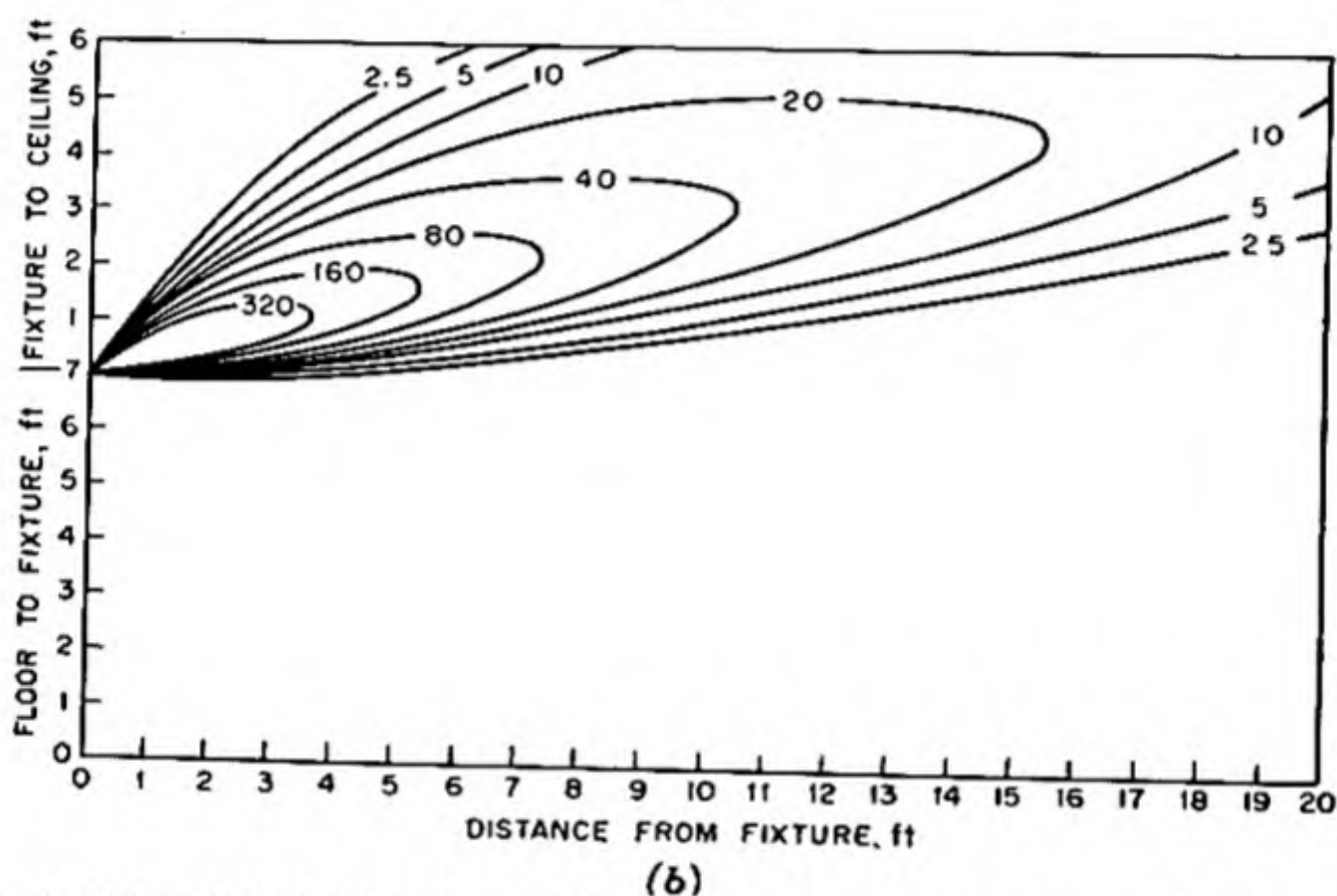
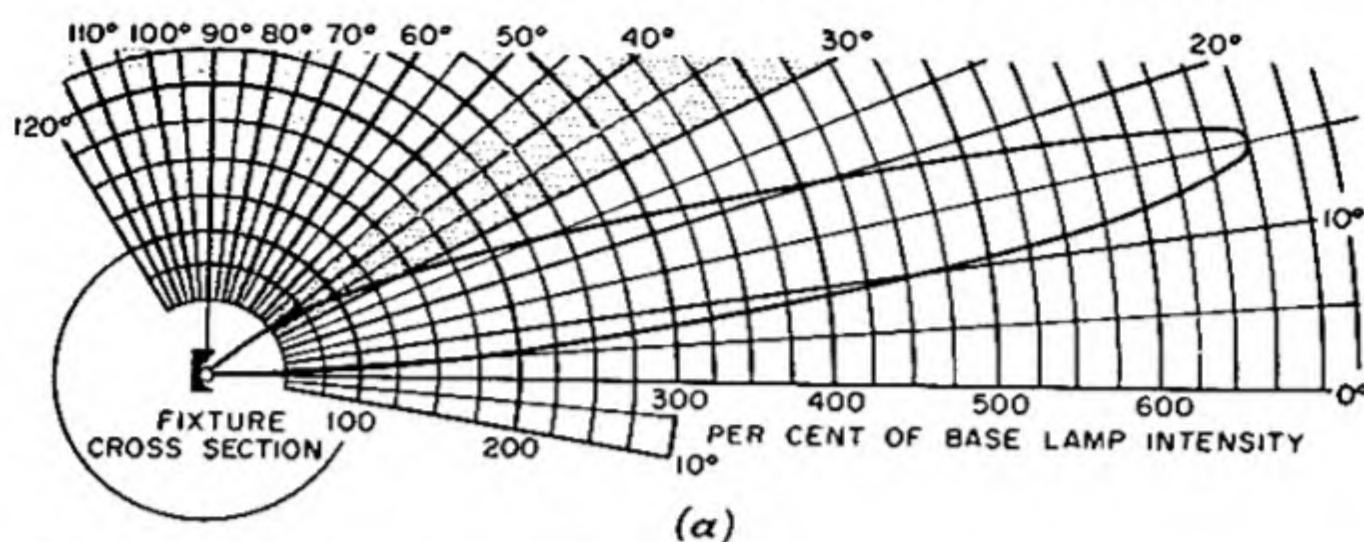


FIG. 2-14. (a) Spatial distribution of ultraviolet from typical bactericidal tubes and louvered reflectors. (b) Isointensity lines in milliwatts per square foot in a plane perpendicular to the center of the G30T8 tube of Fig. 2-9.

Barrier-type units have usually been custom made, but a typical combination unit is described by Fig. 2-15a and b.

#### HEALTH VALUE OF AIR DISINFECTION

Ultraviolet air disinfection is but one of the factors in a complete air sanitation. It is comparable with the removal of dust and noxious vapors. Although air disinfection has been used in industrial applications as such a general sanitary measure, it early came to be thought of as having more specific possible value in preventive medical and public-health applications. Buttolph (1951) has proposed a tentative standard of air sanitation relating ventilation and disinfection to room occupancy. It calls for one air change per minute where there may be as little as 300 cu ft of room volume per occupant. From that it assumes the need for dilution with fresh air or equivalent ultraviolet air disinfection to be proportional to the crowding, to vary directly with the number of occupants in a room and inversely with its volume.



*Hospitals.* When new low-pressure sources of 2537 Å ultraviolet, with a germicidal efficiency fivefold greater than that of previously available sources, revived the interest in practical applications, they were first found by Hart (1936) and Overholt and Betts (1940) to improve the air

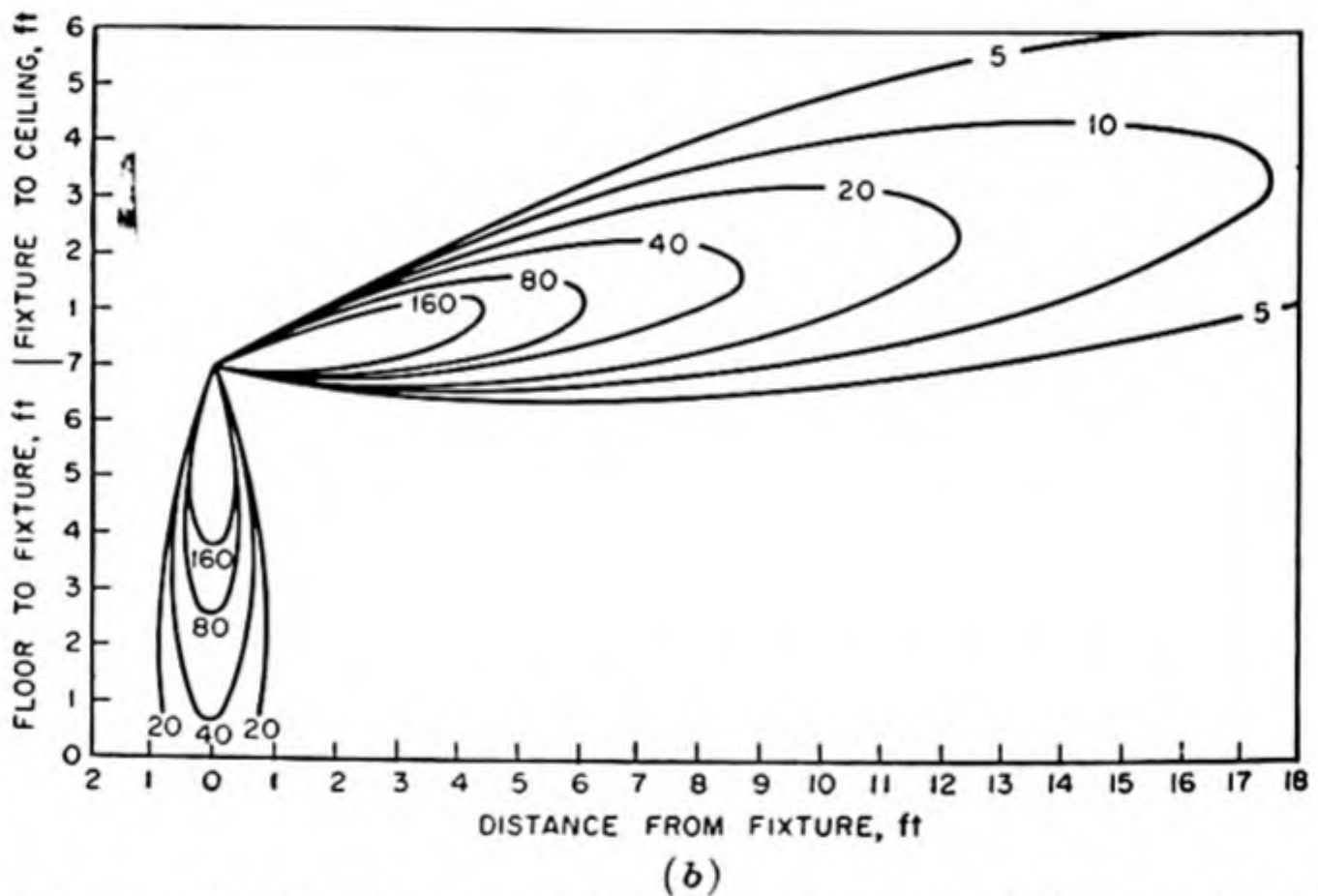
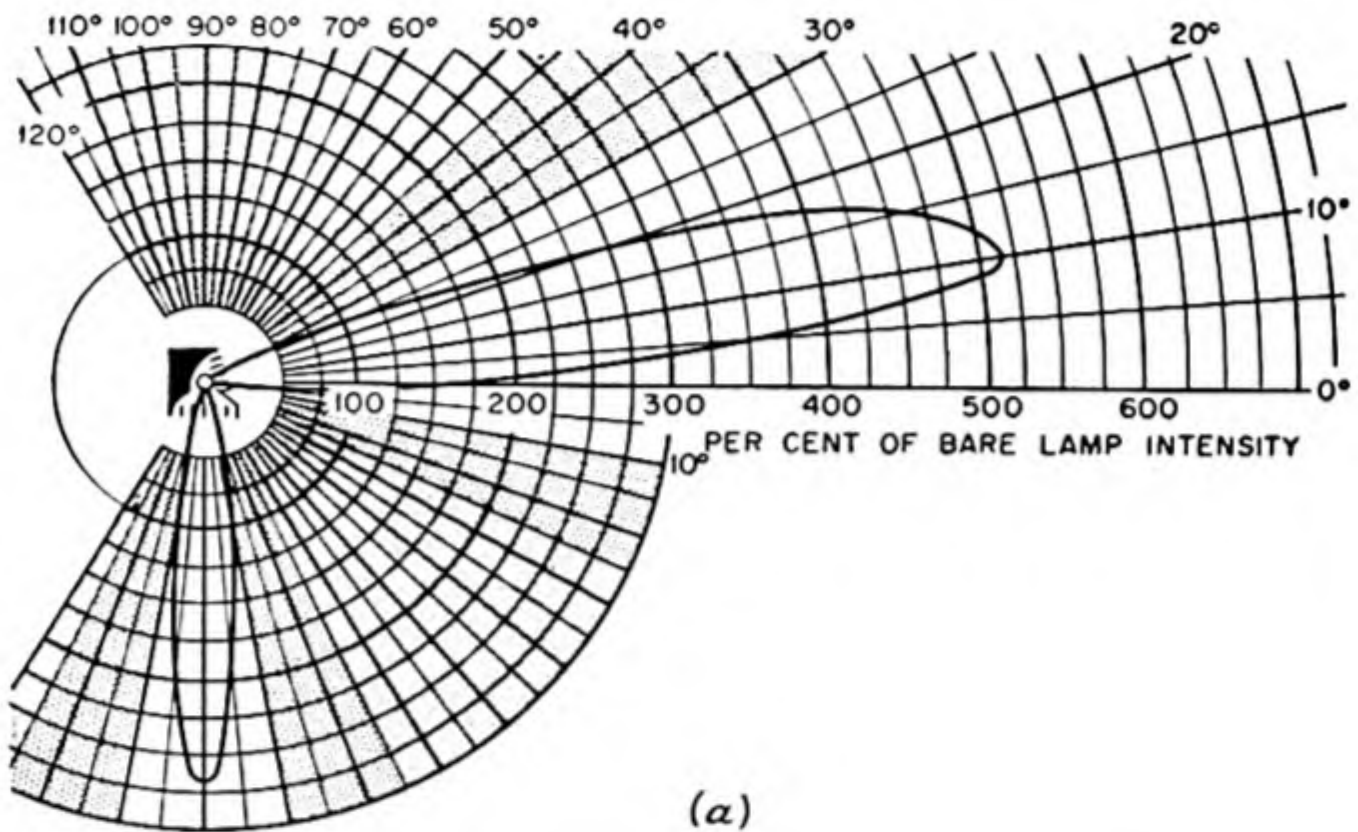


FIG. 2-15. (a) Spatial distribution of ultraviolet from typical bactericidal tubes and louvered reflectors with a downward barrier reflector. (b) Isointensity lines in milliwatts per square foot in a plane perpendicular to the center of the G30T8 tube of Fig. 2-9.

sanitation in operating rooms. This success led to trials by Sauer *et al.* (1942), Del Mundo and McKhann (1941), and Robertson *et al.* (1939; 1943) in infant nurseries where an even more definite improvement was found by air sampling as well as by records of the spread of contagion among infants. On the basis of these tests the use of germicidal lamps

is approved for general hospital use by the American Medical Association (1948).

Lurie (1946) and Vandiviere *et al.* (1949) have shown that tubercle bacilli, either in sputum or air-borne, are readily killed by practical exposures to ultraviolet. Wells and Ratcliffe (1945; Wells *et al.*, 1948) have shown that, in experimental animals, tuberculosis is spread mostly by air-borne organisms so small that they remain suspended in air for long periods. These studies suggest a unique value for ultraviolet air sanitation in tuberculosis hospitals and perhaps even in some homes.

*Schools.* The promising results in hospitals led at once to trials in public schools. Wells *et al.* (1942) and Wells (1945) in Swarthmore and Wells and Holla (1950) in Pleasantville studied the spread of measles and chicken pox as typical of respiratory diseases in general to find that ultraviolet air disinfection suppressed the epidemic occurrence of these diseases in the sense that their incidence was spread out over longer-than-usual time intervals. This modification of the pattern of epidemic spread was thought worth while in spite of some uncertainty as to a significant long-run reduction in the total cases.

Only measles and chicken pox were studied as respiratory diseases, typical in their air-borne manner of spread but atypical in the individual immunity they impart, with the preconception that influenza and the common cold could not be studied directly because of their indefinite diagnosis, their spread in every environment outside the schools, and the almost universal susceptibility to them. As anticipated, air disinfection provided no measurable effect on the incidence of colds and influenza among the school children, and Downs (1950) reported no effect in a surrounding community. The studies by Wells and Holla (1950) showed that measles and chicken pox are too completely typical of respiratory diseases to simplify greatly the study of their epidemiology in schools since two-thirds of their spread occurred outside the school coverage of the ultraviolet installation.

In large consolidated schools served by busses from small towns and the surrounding country, Perkins *et al.* (1947) hoped to study also measles and chicken pox with a minimum spread outside the school environment. There was early indication of some effect on the epidemic spread of the diseases in the schools, but there was also subsequent evidence of their spread in the busses, perhaps enough to blanket the marginal effect of the air disinfection in the school buildings.

Ultraviolet air disinfection is justified in school rooms as a supplement to air sanitation by ventilation, especially in northern latitudes. It has a place as a general sanitary measure along with dust suppression, wash-room sanitation, and habits of personal cleanliness which may be taught and practiced in schools regardless of their effectiveness outside the school.



*Institutions.* Many institutions, through their isolation from surrounding communities, have provided an opportunity to study the possible value of a universal practice of air sanitation in large communities. Schneider *et al.* (1944) early reported a study in a training school for delinquent boys started with such equipment as was then available. DuBuy *et al.* (1948) have since reported no effect on the incidence of disease among the boys, and that air sampling showed little air disinfection. If there was no disinfection, no effect should have been expected, but the more probable explanation is that the air-sampling method did not properly detect the presence or absence of respiratory-disease organisms, that the obsolete equipment did not provide an effective use of the ultraviolet energy even though it was supplied in excess, and that any possible effect on the spread of respiratory diseases in the sleeping rooms was nullified by the lack of separation of the boys in the irradiated dormitory from those in the control dormitory during their class, intimate play, and eating periods.

*Navy Barracks.* In contrast with these results, studies in Navy barracks consistently indicated disinfection of the air and a significant reduction in the spread of general respiratory diseases. Wheeler *et al.* (1945) reported a 25 per cent reduction of respiratory illness and a 50 per cent reduction of the relatively highly resistant saprophytic organisms dominating the air contamination. Miller *et al.* (1948) reported, in a similar but theoretically more effective installation, a 19.2 per cent overall reduction in total respiratory disease and a 24 per cent reduction in the unusually high streptococcus-disease rates. Willmon *et al.* (1948), reviewing four years of Navy barracks study, are less certain of the amount of reduction in disease, and Jarrett *et al.* (1948) reported about 50 per cent reductions in bacteria count but were dissatisfied with the open-plate method which overemphasizes heavier dust-borne contaminants.

*Conservatism as to Value.* The universal appeal of air disinfection as a general sanitary measure and the limited evidence of its specific health value have led committees of the American Public Health Association (1947) and of the National Research Council (1947) to issue warning statements about air disinfection in general and about the ultraviolet method in particular. Both committees point out that, at best, air disinfection can reduce only that limited part of the spread of respiratory disease which may be air-borne, and they emphasize the difficulties in obtaining effective ultraviolet air disinfection without face and skin irritation of room occupants.

There is need for further study of the extent to which air disinfection might supplement the use of face masks, the smothering of the cough and sneeze, and the physical isolation of patients suffering from respiratory disease.

Air disinfection would seem to have most of the possibilities and limitations in health value of unusual amounts of ventilation with outdoor air, amounts ordinarily impractical in the cost of moving and heating. Ultraviolet energy in germicidal barriers across openings would seem to provide a bacterial isolation of rooms and people where doors or glass partitions may be impractical, as in some hospital infant wards. Ultraviolet barriers in air ducts can supplement and in some cases serve the purpose of excessive filtration of duct air, as in pharmaceutical factories.

### FLUID DISINFECTION

When intervening media are gases of negligible absorption and liquids of so great an absorption that the effective penetration distance is negligible, the intensity at the irradiated surface is obviously dominated entirely by the distance through the gas. When, however, there is irradiation through a gas such as air and into the mass of a liquid of low or intermediate absorption, the intensity at any given point in the liquid is determined primarily by the total distance from the source to the point, and secondarily by the absorption from the surface of the liquid to the same point. This absorption can vary over a 10,000-fold range, from water of low iron content which can be disinfected in a duct in much the same way as air, when due allowances are made for its greater absorption and the increased ultraviolet tolerance of wet bacteria, to milk and serum which must be processed in films of thicknesses less than a few thousandths of an inch.

### WATER DISINFECTION

Water was the first liquid to be disinfected by ultraviolet and with commercial equipment using high-pressure mercury arcs. The method could not compare with chlorine disinfection, economically, and it did not provide any evidence of effective use as does "residual chlorine." Although low-pressure sources of germicidal ultraviolet have greatly reduced the cost, ultraviolet is now used only in instances where chemical methods or boiling cannot be tolerated and where there may be routine bacteriological control, such as in beverage, food, and pharmaceutical processes.

Water-borne *E. coli* require an 8–10 times greater ultraviolet exposure for a given kill than when air-borne, 150–200 ultraviolet  $\mu\text{w-min/cm}^2$  for a theoretical 99 per cent kill in distilled water (Luckiesh and Holladay, 1944). The ultraviolet absorption of all water is much greater than that of air and of itself varies more than tenfold from a 90 per cent absorption in 5 in. to the same absorption in 50 in. (Fig. 2-16, from data by Luckiesh *et al.*, 1947, 1949). This variation is apparently almost entirely due to dissolved iron salts. Since water readily acquires sufficient iron for such variations in absorption from contact with iron pipes and storage tanks,



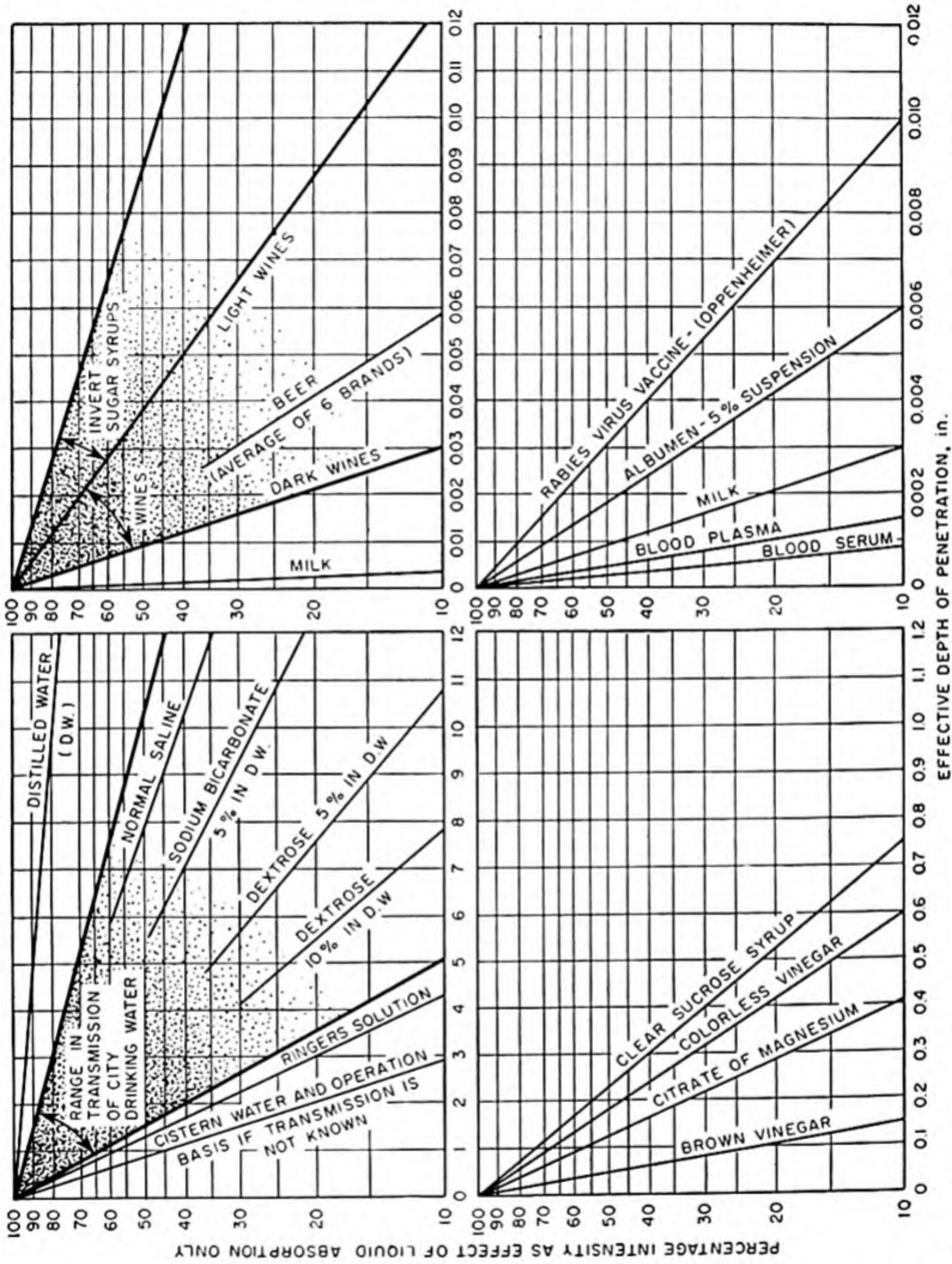


Fig. 2-16. Effective depth of penetration of 2537 Å, 90 per cent absorption, into various liquids. (Luckiesh *et al.*, 1944.)

regardless of its previous condition, water-disinfecting equipment must be designed to ensure adequate treatment of the most absorptive water, with some provision for effective use also with water of considerably less absorption. Because of these factors, water disinfection must be based on exposures of the order of 700–1000 ultraviolet  $\mu\text{W-min/cm}^2$ .

*Water-disinfecting Devices.* Ultraviolet water-disinfecting devices fall into three general classes: (1) devices in which water under pressure is disinfected by lamps either immersed in the water or separated from it by a concentric jacket, (2) devices in which the lamps are isolated in an air-pressure chamber over the surface of the water, and (3) devices which

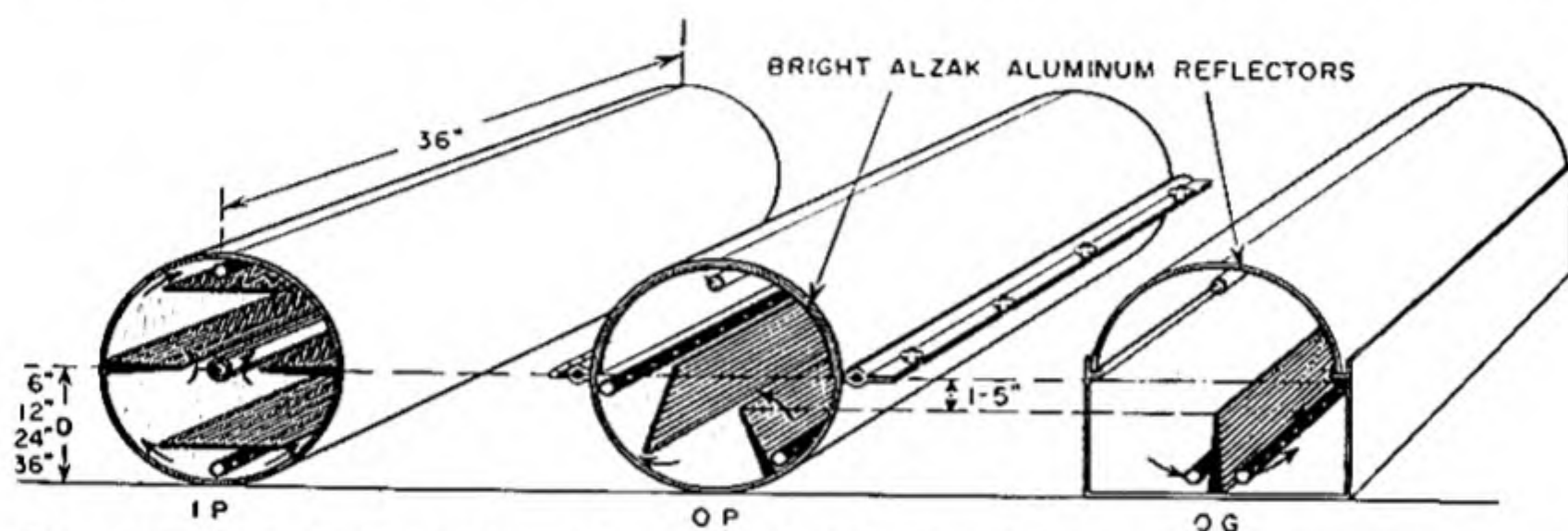


FIG. 2-17. Basic types of ultraviolet water-disinfecting devices. I.P., source immersed in water under pressure. O.P., source in air over water, subject to air pressure and gravity flow. O.G., open gravity type.

provide for disinfection of free-flowing water in a trough over which ultraviolet sources may be suspended. The open gravity type is used in factory processes involving small quantities of water and in isolated camp and farm installations where there may already have been a routine bacteriological control. A variety of pressure-type units have been developed commercially, usually with considerable difficulty, where the ultraviolet source has been entirely surrounded by water but isolated from it by a concentric ultraviolet-transmitting jacket because of ultraviolet-absorbing deposits on the lamp or its surrounding jacket. Complete immersion of the ultraviolet source is usually impractical also because of the excessive cooling of the mercury arc. The more complicated but more promising method isolates the ultraviolet source under air pressure and over the water in the remaining half or two-thirds of the tank. Examples of these typical devices are shown schematically in Fig. 2-17.

#### ABSORPTIVE LIQUIDS

Since water was first disinfected by ultraviolet with commercial equipment, reported by Recklinghausen (1914) and the entire subject reviewed in a U.S. Public Health Service report (1920), attempts have been made to disinfect more absorptive liquids such as wine, beer, and milk. The



method has seemed even more attractive for pharmaceutical liquids such as vaccines, serums, blood, and blood plasma, to which pasteurizing temperatures may be destructive. Such liquids are much more absorptive of ultraviolet than is water.

*Effective Depth of Penetration.* Absorption of ultraviolet by these liquids is expressed in Fig. 2-16, in practical terms of the depth or film thickness through which there is a 90 per cent absorption of 2537 Å ultraviolet. This "effective depth of penetration" can, however, be made fully effective only in film-spreading devices which ensure enough turbulence in the film to expose all particles to the full range of ultraviolet intensities through the depth of the film during the exposure time.

*Film Spreaders.* Liquids with effective depths of penetration less than 0.1 in. must be irradiated in special equipment which will expose those liquids in moving layers of less than this depth. With the more absorptive or more viscous liquids, films of controlled thickness cannot be produced by gravity, and it is necessary to resort to centrifugal methods or to the wetting of moving surfaces from which the liquids are removed after irradiation. Some of the many possible thin-film irradiators are suggested schematically in Fig. 2-18. The film thickness and rate of flow (exposure time) is by gravity and limited to it. In type C, both thickness and flow are by centrifugal force and thus are subject to considerable control. In type D, the film flow is by gravity and the slope of the cylinder, while the film is spread by centrifugal force, the latter to some extent decreasing the gravity flow. Type E is almost entirely dependent on adhesion and viscosity for the film formation and is inherently limited as to the thinness of film formed. Type C provides for short-time irradiation of thinner films, but with difficulty in providing the required high ultraviolet intensity. Types D and E provide for longer time, lower intensity irradiation, but with difficulty in providing sufficiently thin films of the more absorptive liquids. In types B, C, and D, the irradiated liquid may be protected from contact with air and ozone by filling the irradiators with a neutral gas such as nitrogen or carbon dioxide. In types C and E, the thickness of the irradiated film can be controlled, regardless of viscosity, by the use of mechanical film spreaders. Type F defines the film thickness between flat plates of fused-quartz glass and the exposure time by the rate of flow. All but type F have the time-proved feature of irradiation of and through the free-flowing and continually renewed surface of the irradiated liquid. Type F has the important feature of being inherently a closed system adaptable to continuous pressure operation. The slower moving liquid surface in contact with the device is exposed for a longer time to compensate partially for the reduction of the ultraviolet intensity by the absorption of the liquid. Deposits of the nature of polymerization products, which usually form on the film contact surfaces of these devices, are less objectionable

in all types other than F. Types C, D, E, and F have recently been made commercially available, primarily for the inactivation of serums and vaccines. Types A and B have been adapted to milk disinfection.

*Laboratory Methods.* Useful laboratory methods for the ultraviolet disinfection of absorptive liquids have been reported. Hollaender and Oliphant (1946) have used a spherical fused-quartz glass flask, inclined about  $45^\circ$  from the vertical and about one-third full of liquid, rotated slowly on its axis. The film of liquid which was dragged up and over on

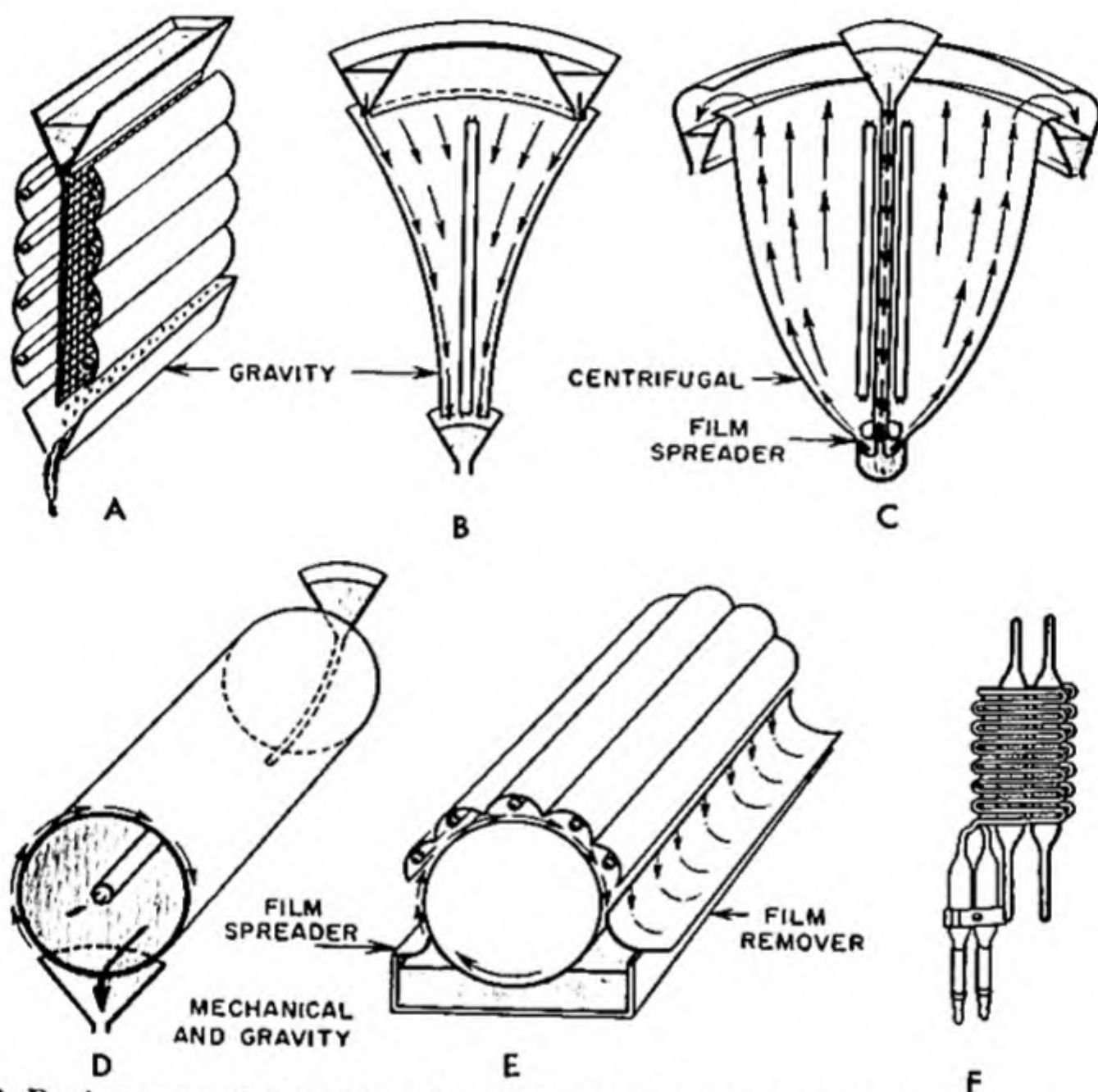


FIG. 2-18. Basic types of thin-film ultraviolet irradiators for relatively opaque liquids.

the inner side of the lower two-thirds of the flask was irradiated through the upper third by ultraviolet sources surrounding the flask; this provided a closed system but only for intermittent operation. Hollaender has also suggested an inclined fused-quartz tube containing indentations along its lower third and paralleled above by three or more tubular ultraviolet sources which irradiate a turbulent stream of liquid through the uncoated upper two-thirds of the tube. This is a closed system without trouble from deposits and is adaptable to continuous pressure operation. It would seem to have practical possibilities in spite of its inefficient use of the ultraviolet sources.



*Effective Exposures.* Even with absorption minimized by the thinnest films provided in centrifugal devices, exposures at least as great as for water-borne organisms would be required: 200–300 ultraviolet  $\mu\text{w-min/cm}^2$ . The minimum exposure for the thicker films provided by gravity devices might be increased severalfold to 700–1000 ultraviolet  $\mu\text{w-min/cm}^2$  such as is required in the disinfection of absorptive water through several inches of depth. Since it is difficult to provide ultraviolet intensities greater than 10,000 ultraviolet  $\mu\text{w/cm}^2$  (10 ultraviolet watts/sq ft), the exposure times in such devices should not be less than 1–2 sec for the thinnest films to 4–6 sec for the thickest.

Although the speed of 500–1000 rpm of the centrifugal devices provides linear film speeds of 200–400 ft/min, this is in a helical, nearly circular path. Only the relatively slow, forward component contributes to the exposure time. The centrifugal force provides this forward component directly only in rotors essentially conical in shape (Fig. 2-18C). In a cylindrical rotor the centrifugal force spreads the film in both directions perpendicular to the helical path, but there is effective movement of the film as a whole only in so far as the gravity-pressure equilibrium is disturbed by the delivery of liquid from the film and only in so far as the rotor is so inclined as to permit gravity flow. Note also in this connection that the rate of liquid flow through these devices defines the exposure time only through the amount actually and momentarily being processed in the film, an amount usually very difficult to measure accurately.

*Operating Controls.* Where there may be variations in ultraviolet absorption or penetration into an irradiated film such as are shown in Fig. 2-16, there must be provision in commercial devices by which the film thickness and the ultraviolet exposure are completely controlled by the liquid absorption. The ideal control would provide an automatic adjustment of the exposure to the absorption with the film thickness held constant mechanically. The exposure should, in turn, be based directly on the ultraviolet intensity at the film surface, rather than on the electrical characteristics of the sources, to compensate for their output depreciation. Fast-acting relays and valves should stop the delivery of material instantly upon the shortest power failure and should provide for rejection or reprocessing of material remaining in the device.

#### DISINFECTION OF SURFACES OF GRANULAR MATERIALS

There is practically no penetration or reflection of ultraviolet energy in the irradiation of granulated or powdered materials. Only the upper fourth or fifth of the surface of individual particles can be irradiated at any given instant of time. However actively stirred or agitated, the surface of such particles is therefore effectively irradiated only one-fourth to one-fifth of the time. The particles shade each other as soon as there is a layer more than one particle thick, so that there is a "coefficient of

shading" similar to the coefficient of absorption of liquids which defines the "effective depth of agitation" for granular materials, analogous to the effective depth of penetration of water. Experience indicates that the effective depth of agitation is of the order of ten times the diameter of granulated products.

The theory of treating granulated and powdered solids is practically the same as for highly absorptive liquids, with effective depths of agitation of the order of the effective depth of penetration into sugar syrups. Although the bacteria on the surface of dry sugar crystals, for example, may be about ten times as susceptible to ultraviolet killing as are those in water, this is offset by the presence of the individual sugar crystals on which the bacteria ride at the surface of the layer for only about a tenth of the time. When the crystals are in the irradiated layer, bacteria on them are irradiated only about a fifth of the time on the exposed sides of the moving crystals. The result is that granular particles can be disinfected with only about one-fifth the efficiency of water disinfection and that the method is not practical on powdery materials. The disinfection of the surfaces of granular materials is well illustrated by the commercial method used on canner's sugar.

#### SUGAR

Thermoduric bacteria survive the vacuum evaporator temperatures of sugar-syrup concentration and, rejected by the sugar crystals during formation, remain in the final film of dilute syrup left on the crystal surfaces. Ordinarily harmless, they may cause serious spoilage in canned foods and beverages.

Such sugar, preferably in coarse crystals in a layer of about  $\frac{1}{4}$  in. at rest, is continually vibrated, stirred, or cascaded on a conveyor under closely spaced germicidal lamps (Fig. 2-10) providing of the order of 23,000 ultraviolet mw/sq ft of conveyor surface. The length and speed of the conveyor may be such as to provide a total exposure time of 15-5 sec for an exposure of the order of 500 mw-min/sq ft.

#### GRAINS AND SMOOTH-SKIN FRUIT

An ultraviolet method has been reported by Ewest and Leicher (1939) to be effective in reducing the superficial mold contamination of hard grains such as that which develops after storage in the tropics. A similar but simpler method is reported by Matelsky (1950) as effective on smooth-skinned fruit such as cherries.

#### ULTRAVIOLET-INDUCED MUTANTS FOR NEW FUNGI

The use of ultraviolet to produce mutants of fungi in a search for new or better commercial characteristics deserves mention because of its



novelty rather than its commercial importance. Emmons and Hollaender (1939) showed that the curve which represents the efficiency of different wave lengths of ultraviolet producing mutations in fungi parallels closely the germicidal-action curve. Hollaender and Emmons (1941, 1946) correlated mutants of fungi produced by ultraviolet irradiation with the naturally occurring species probably produced by sunlight. From this, techniques have been developed for obtaining mutants, for example, for better yields of citric and itaconic acids and of penicillin and the similar antibiotics (see chapters on bacteria and fungi).

### PROTECTION AND PROCESSING OF PRODUCTS

Because of its high germicidal effectiveness compared with its other photochemical, erythema, and thermal effects, ultraviolet energy, especially of wave length 2537 Å, has been used for the protection and disinfection of many products of so unstable a composition as to prohibit the use of more conventional methods. Such applications have developed in food, pharmaceutical, and beverage processing and storage places.

### MOLD, ANTIBIOTICS, AND PARENTERAL FLUIDS

The mold-derived antibiotics and many parenteral liquids are very susceptible to contamination by the normal mold and bacterial content of air. This contamination is often of a chemical nature precluding terminal sterilization by heat. Some serums and antitoxins are developed by the growth of bacteria and viruses which must be finally inactivated by methods which will not at the same time destroy the desirable properties of the preparation. Ultraviolet has served to protect such materials during processing and to provide a final inactivation where controlled bacterial growth has been a part of the process. The germicidal lamps are used in ducts and hoods, over work tables, for upper-air disinfection, and also for thin-film irradiation (Fig. 2-18) by the methods previously discussed.

### BLOOD PLASMA

One of the more unusual applications of ultraviolet is for the disinfection of blood plasma of the hepatitis virus. Several commercial devices have been developed. All provide for irradiation in films of the order of  $\frac{1}{1000}$  in. in thickness or for the violent agitation of somewhat thicker films. It is important to remember that, because of the low penetration of the ultraviolet, changes in film thickness, even of microscopic dimensions, may seriously interfere with any of these methods. These methods and devices have already been discussed generally for absorptive liquids (Figs. 2-16 and 18). Preliminary to the use of any

of these methods, there must be a complete removal by centrifuging or filtration of all clumps down to an empirically determined and specified size. A machine or process should produce a specified degree of sterility in test runs of plasma contaminated with a test organism of an ultraviolet-exposure tolerance comparable with that of the hepatitis virus. *Sarcina lutea*, whose packet growth habit may simulate the minute clumps remaining in plasma after clarification, is suggested.

In operation there should be a continuous record of plasma flow and ultraviolet intensity, similar to the controls used in continuous methods of milk pasteurization. A cadmium photocell (Figs. 2-1 and 5b), described by Taylor and Haynes (1947), and a recording microammeter are suitable for the ultraviolet control. The rate of flow may be controlled by a pump with variable-speed drive. Rapidly operating relays and electric valves should stop or divert the delivery of plasma from an intermediate storage reservoir of a capacity much greater than that of the irradiating device.

#### SYRUP, FRUIT-JUICE, AND WINE STORAGE

The sugar content of sugar and fruit syrups is usually such as to prevent fermentation even though a mold scum may form on exposed surfaces. Whenever there is condensation of moisture on the sides and tops of syrup-storage tanks, it may dilute the surface layer of the stored syrup enough to permit destructive and otherwise very objectionable fermentation in addition to the usual mold formation. Germicidal tubes are being used to prevent such fermentation and mold on the surfaces of tank-stored sugar and fruit-juice syrups used for soft drinks and confectionery. Continuous irradiation with an intensity of at least 5 ultraviolet mw/sq ft is required.

#### MEAT STORAGE

The most extensive single industrial use of germicidal lamps is to reduce the growth of bacteria and molds on the surface of meat and on shelves, walls, and floors of retail-meat-storage refrigerators operated at 35°-45°F. There is little need for such provisions in cold-storage rooms operated below zero, nor has there been any such need in the larger meat-processing factories where exact control of temperature, humidity, and air movement produces similar results. Proper use of the ultraviolet does not take the place of established periodic sanitary maintenance but does supplement it by a continuous suppression of spoilage and odors. The ultraviolet intensities required are of the order of only the 5 mw/sq ft effective for mold suppression in other applications.

Ultraviolet is effectively used to suppress surface slime molds on meat stored for 3 days at a temperature of about 60°F for rapid aging or tenderization. Contrary to the impression of some, the ultraviolet has no direct effect on the enzymatic tenderizing.



TABLE 2-6. CHARACTERISTICS OF MEDIUM- AND HIGH-PRESSURE MERCURY-VAPOR SOURCES OF ULTRAVIOLET ENERGY<sup>a</sup>

Designation	Rated power input, watts	Ultraviolet output, UV watts					Visible-energy output, 3800-7600 Å, UV watts	Maximum over-all length, in.	Useful arc length, in.	Maximum diameter, in.	Open circuit voltage, v	Potential drop voltage, v	Operating lamp current, amp	Rated average life <sup>c</sup>
		Far UV (2200-2500 Å)	Far UV (2500-2800 Å)	Middle UV (2800-3200 Å)	Bactericidal action 2650 Å equivalence <sup>b</sup> of 2200-3200 Å	Near UV (3200-3600 Å)	Near UV (3600-3800 Å)							
C-H3 <sup>d,e</sup>	85	0.46	1.50	4.60	2.59	0.93	2.84	6.0	1 1/16	1.25	440	250	0.4	500
A-H4 <sup>d,e,f</sup>	100	.....	.....	0.72	0.02	0.92	3.39	10.4	1	1.25	250	130	0.9	1000
B-H4 <sup>d,e,f</sup>	100	.....	.....	0.001	.....	0.20	0.63	0.1	1	2.00	250	130	0.9	1000
C-H4 <sup>d,e,f</sup>	100	.....	.....	0.05	.....	0.43	1.77	7.8	1	4.62	250	130	0.9	1000
E-H4	100	.....	.....	0.005	.....	0.36	6.22	33.1	1 5/8	1.73	250	135	2.1	3000 <sup>g</sup>
A-H5 <sup>e,f</sup>	250	.....	.....	10.36	10.0	1.28	7.19	18.8	3	0.75	225	92	3.1	3000 <sup>g</sup>
UA-2 <sup>d,e</sup>	250	3.67	8.14	13.25	13.0	1.56	9.13	24.9	6	0.75	260	135	3.1	3000 <sup>g</sup>
UA-3 <sup>d,e</sup>	360	4.53	10.31	2.8	.....	3	14.4	55.0	2 3/4	4 5/8	220	135	3.2	4000 <sup>h</sup>
E-H1 <sup>d,e,f</sup>	400	.....	.....	7.0	.....	7.5	36.0	143.0	5 3/8	7	460	265	4.0	4000 <sup>h</sup>
A-H15 <sup>d,e</sup>	1000	.....	.....	6.8	4.0	9.0	53.0	290.0	1	0.25	1250	840	1.4	1000
A-H6 <sup>d,i</sup>	1000	.....	.....	75.0	40.0	13.0	79.0	290.0	17 3/4	0.75	750	450	3.1	1000
B-H6 <sup>d</sup>	1000	10.0	21.0	90.0	80.0	10.0	55.0	126.0	48	0.50	2650	2000	1.58	1000
UA-11 <sup>d,e</sup>	1200	25.0	60.0	230	270	25	150	310	49	1.19	245	125	10.5	1000 <sup>k</sup>
UA-15	3000	100.0	205	0.90	0.03	0.22	6.33	40.5	50	1.19	850	535	6.1	3000 <sup>l</sup>
UA-4 <sup>d,e</sup>	1200	.....	.....	.....	.....	0.24	21.32	357.4	64	1.19	1100	660	6.1	1000
B-H9 <sup>e,f</sup>	3000	.....	.....	.....	.....	3.77	71.62	361.0						
UA-7 <sup>d,e</sup>	3750	.....	.....	3.89	0.14									

<sup>a</sup> May be burned in any position, except A-H6 which is designed for horizontal use only. All outer bulbs have clear finish except B-H4, which has natural red purple, and C-H4 and E-H4, which have aluminum reflectors.

<sup>b</sup> Multiply by 0.85 for the bactericidal equivalence of 2537 Å in ultraviolet watts.

<sup>c</sup> 5 hr per start.

<sup>d</sup> General Electric Company designation.

<sup>e</sup> Hanovia Chemical & Manufacturing Co. designation.

<sup>f</sup> Westinghouse Electric Corporation designation.

<sup>g</sup> Life, 1000 hr with special operating conditions.

<sup>h</sup> The life may be somewhat impaired if the fixture exceeds specified temperature limits.

<sup>i</sup> A-H6 requires water jacket; B-H6 requires air jet cooling.

<sup>j</sup> Life, 75 hr with special operating conditions.

<sup>k</sup> Requires an auxiliary device for starting and restarting.

<sup>l</sup> Life, 10,000 hours with special operating conditions.

<sup>m</sup> Only single bulb used.

## HIGHER PRESSURE MERCURY SOURCES OF ULTRAVIOLET

Electric-discharge mercury lamps in tubes of high ultraviolet transmission develop relatively full-line spectra when operated at higher (over 400 mm or 0.5 atm) pressure. The total ultraviolet per watt of electrical input drops to about one-half that of a low-pressure lamp in tubes of the same transmission. The energy is distributed variably among about 20 lines instead of being almost entirely concentrated at 2537 Å, as shown graphically in Fig. 2-5. Lines other than 2537 Å of good energy content become very useful for studies of biological effects as functions of wave length. The high intrinsic output of the higher pressure sources becomes essential for effects requiring ultraviolet intensities unobtainable even at the surface of the low-pressure sources. High-pressure lamps in the lower wattage sizes are essential to any research involving isolation of spectral lines or bands by optical methods. Their adaptability can be inferred from the source dimensions given in Tables 2-3 and 6, based partially on the IES Lighting Handbook, 2d ed. (1952).

### INTENSITY AND VARIATIONS WITH DISTANCE

The radiant-energy intensity at a distance of 1 meter, in microwatts per square centimeter, can be approximated from the total watts output rating of Table 2-6 by multiplying by a factor of 10. Conversion to other units and distances can be made by the methods outlined for low-pressure sources.

### INDIVIDUAL LINE INTENSITIES

Persons who need a more detailed analysis of the line spectra of the sources listed in Tables 2-3 and 6 but who do not have facilities for making line-intensity measurements under the actual conditions of their experimentation should correspond directly with manufacturers of the source being used. The relative energy distribution among the lines of high-pressure mercury lamps varies between individuals and between groups in such a complicated fashion that no general rules can be given and detailed listings here become impractical. A fairly representative high-pressure spectrum is shown graphically in Fig. 2-5 and is typical of such lamps as the UA-3 and UA-11 of Table 2-6.

### STARTING AND RESTARTING TIMES

With few exceptions, the higher pressure lamps start with low-pressure characteristics and require 3-5 min to reach normal operating temperature, pressure, and radiation output. If momentarily extinguished, they also require a cooling and restarting period equal to or in some cases one-half longer than their warm-up time.



## LIFE AND DEPRECIATION

Life ratings of all electric-discharge lamps involve complicated factors of economy as well as physical mortality. The burn-out life is usually that at which one-half the lamps of a test group reach a physical or economic end of life. The latter is very dependent on variable factors of lamp and electric costs and the nature of the application. For operating intervals of less than 5 hr the life decreases rapidly with the length of the operating interval. For longer operating intervals the total life may increase to several times the 5-hr interval rating. The general form of the depreciation curve is that of Fig. 2-11.

An important variable in the depreciation of all higher pressure mercury arcs is the "solarization" effect of the ultraviolet and heat on the transmission of the fused quartz generally used. The effect increases as the wave length decreases and may reduce the output of 2200–2500 Å energy to one-third the initial and of 2500–2800 Å to one-half the initial in a few hours of operation. This accounts for some of the discrepancies in published data on the shorter ultraviolet output of commercial sources. The effect is relatively small at wave lengths longer than 2800 Å.

## RESEARCH DETERMINATION OF OUTPUT AND INTENSITY

As was emphasized for low-pressure sources, although their commercial radiation ratings should be fully specified in all research reports, these ratings can be considered only a first approximation to the energy output and intensity actually effective in any specific research. Whenever direct measurements of energy at the point of application are impractical, calibrated lamps should be used. The intensities that they provide can be calculated for various distances by the methods outlined for low-pressure sources as long as there are no intervening optical systems or filters.

In work at wave lengths less than 2800 Å, and especially with the higher pressure sources, the source itself should be frequently calibrated for output where direct measurements of the irradiated surfaces of material are in practice.

## MERCURY-AMALGAM AND OTHER METAL ARCS

Laboratory workers generally fabricate their own electric-discharge metal-vapor arcs, other than mercury, although such lamps have been occasionally imported for sale in the United States. Various commercial types available in the past are described by Meyer and Seitz (1949). Their availability is too uncertain and their radiation characteristics are too unstandardized for inclusion in this chapter. It should be noted here, however, that cadmium-amalgam lamps providing low-intensity cadmium lines of wave lengths greater than 3000 Å are commercially

TABLE 2-7. SUNLAMPS

Designation	Rated power output, watts	Ultraviolet output, (UV watts)					Visible-energy output, 3800-7600 Å, UV watts	Maximum over-all length, in.	Useful arc length, in.	Maximum diameter, in.	Open circuit voltage, v	Potential drop voltage, v	Operating lamp current, amp	Rated average life <sup>a,b</sup>
		Far UV (2500-2800 Å)	Middle UV (2800-3200 Å)	Bactericidal-action 2650 Å equivalence of 2500-3200 Å	Erythmal-action 2967 Å equivalence of 2500-3200 Å	Near UV (3200-3800 Å)								
S-1	400	0.01	3.2	0.08	0.68	4.5	45	6.5	0.5	2.8	33	14	30	750
S-4	100	0.01	2.1	0.07	0.50	3.6	12	6.8	1	2.6	245	130	0.9	1000
RS-4 <sup>c</sup>	100	0.01	1.5	0.06	0.35	2.9	9.6	5.3	1	5	245	130	0.9	1000
RS <sup>c</sup>	275	....	0.5	0.03	0.35	2.9	8.1	7	1.6	5	<sup>d</sup>	<sup>d</sup>	<sup>d</sup>	1000
FS-20 <sup>e</sup>	20	....	2.0	0.08	0.40	1.4	1.0	24	20	1.5	118	57	0.37	4000
FS-40 <sup>e</sup>	40	....	5.0	0.24	1.00	3.5	2.4	48	44	1.5	200	103	0.42	4000

<sup>a</sup> 5 hr per start.<sup>b</sup> Fluorescent sunlamps are also rated at 5000 15-min applications; RS lamps at 600-1000 15-min applications; S-1 at 800 15-min applications; S-4 and RS-4 at 1000 15-min applications.<sup>c</sup> These lamps contain their own internal reflectors; output figures apply to the energy directed out the face of the lamp.<sup>d</sup> This lamp has a tungsten-filament resistance and a thermal switch enclosed in its reflector-type outer envelope. It is operated without external ballast on 110- to 125-volt, 50- to 60-cycle alternating current only.<sup>e</sup> Preheat starting fluorescent sunlamp, output data approximate.



available as are also similar potassium and sodium lamps. None of them are, however, sources of ultraviolet of the wave lengths generally desired in biological work.

### SUNLAMPS

Commercial sunlamps have been used for some biological research. They provide energy down to 2800 Å from sources of sufficiently high intrinsic intensity to adapt them to optical methods of isolating narrow spectral regions. They have the general characteristics of high-pressure quartz-mercury arcs with the radiation limited to about 2800 Å by the enclosing bulbs or tubes. Laboratory housings for the S-4 lamp have been commercially available. Lamp CH-3 of Table 2-6 is proving more

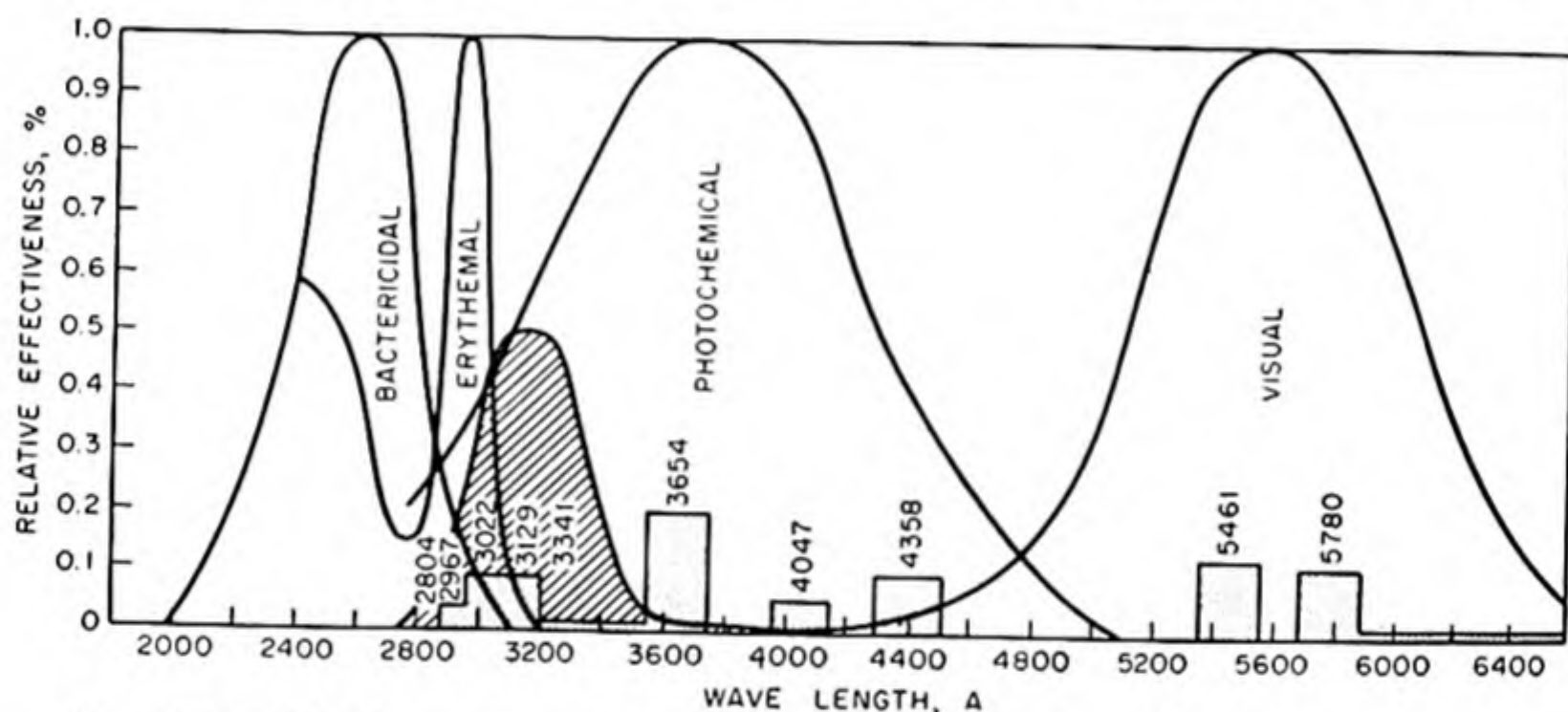


FIG. 2-19. Relative energy distribution of fluorescent and S-4 type sunlamps.

useful in the same devices because it provides essentially the full quartz-arc spectrum. Table 2-7 permits comparison with sources of Tables 2-3 and 6. Figure 2-19, which shows the relative energy distribution of sunlamps, is comparable with Fig. 2-5. The S-1 lamp spectrum is similar to that of the S-4 but with addition of a continuous visible and infrared spectrum from a tungsten filament in multiple with the mercury arc and operating at a temperature of about 2000°K. The RS spectrum has similar visible and infrared components from a tungsten filament in series with the arc.

As indicated in Fig. 2-19, fluorescent sunlamps provide a continuous ultraviolet spectrum with a peak at about 3100 Å. Mercury-arc lines transmitted by the phosphor make an insignificant addition to it. The generated energy in these lines is comparable with that in the lines of the germicidal lamp and is transmitted to an extent of 60–70 per cent by the phosphor. Although the low emission intensity of these lamps makes them unsuitable for use with optical isolating systems, they are comparable with the high-pressure mercury arcs for low-intensity general irradiation with energy of wave lengths in the 3000–3200 Å range. They are

unique sources of energy in the 3200-3400 Å region of the ultraviolet spectrum.

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## CHAPTER 3

### Sunlight as a Source of Radiation

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*The sun as a radiator. Sunlight on top of the atmosphere. Solar ultraviolet radiation at the earth's surface. Atmospheric ozone. Calculated ultraviolet intensity at the earth's surface. Observed ultraviolet intensity at the earth's surface. Solar infrared spectrum at the earth's surface. References.*

#### THE SUN AS A RADIATOR

As a star, the sun is quite ordinary; it is placed by astronomers in spectral class  $G_0$  of yellow stars which, on the average, are about one one-hundredth as bright as average blue stars and a hundred times brighter than average red stars. It is located near the middle of the main sequence into which stars fall when absolute magnitudes are plotted against spectral class. As the source of a spectrum of radiations extending from X-ray to radio wave lengths which fall on the earth, conveniently situated to utilize them in sustenance of life, it is a fascinating object of never-ending study. The story of the sun has been well written by Abetti (1938), by Menzel (1949), and by Hoyle (1949). Hoyle devoted his effort to specialized problems of solar physics, while Abetti and Menzel gave more general discussions of knowledge of the sun. The radiations of the sun probably derive their energy from nuclear reactions deep in the gaseous interior, where the temperature is about 40 million degrees and the density is 76 compared with a density of 1.4 for the entire sun. These radiations finally emerge from the outer layers, mainly at temperatures of  $4000^{\circ}$ – $6000^{\circ}$ K, and then travel unmodified for 8 brief minutes until they reach the surface of the earth, at which point they are the principal interest of this chapter.

Since the sun is gaseous throughout, transitions in temperature, the states of matter, and the character of the radiation emitted are gradual along the radius. Yet several marked distinctions in these properties exist, which permit the division of the outer reaches of the incandescent mass of gas into several regions, each characterized by its radiative properties. The photosphere, or light sphere, is the innermost and most



sharply defined of these transitional regions. When viewed in visible light the photosphere appears to be a sharply defined disk,  $1.39 \times 10^6$  km in diameter, considerably brighter at the center than at the edge, or limb. It is the source of the continuous spectrum of the sun, or rather, the region in the sun in which predominantly continuous emission changes rather abruptly into emission of spectral lines. This transition, owing to a rapid decrease in opacity of the solar gases in the region of the photosphere, takes place in a few hundred kilometers in a level where the pressure is about  $10^{-1}$  atmosphere.

The transmission coefficient  $t_\lambda$  of a homogeneous layer of absorbing gas for light of wave length  $\lambda$  is given by

$$t_\lambda = e^{-\alpha_\lambda h} \quad (3-1)$$

where  $\alpha_\lambda$  = the absorption coefficient for wave length  $\lambda$  and  $h$  = the thickness of the absorbing layer. The sharpness of the photosphere depends on the circumstance that, for visible light, the values of  $\alpha_\lambda h$  are sufficiently high that a relatively thin layer  $h$  of the gases is opaque; that is, the product  $\alpha_\lambda h$  is very great. Relative to any comparison wave length, say, in the visible region, radiation emerging from the sun at other wave lengths where  $\alpha_\lambda$  is greater will come from higher and therefore cooler layers, and the intensity will be lower. At wave lengths where  $\alpha_\lambda$  is smaller, a thicker layer of the sun is required to be opaque, and the radiations which escape come from a region of higher temperature and are, accordingly, relatively more intense. Thus a part of the visible and near-infrared continuum of the photosphere matches the spectrum of a  $6000^\circ\text{K}$  black body rather well, whereas the blue and ultraviolet intensities lie considerably below a  $6000^\circ\text{K}$  source, and there is evidence that the infrared spectrum in the 8- to  $13\text{-}\mu$  region is fitted better by a  $7000^\circ\text{K}$  intensity curve.

Although the photosphere is sharp in visible light, it is not uniformly bright along the diameter, the center of the disk being considerably brighter than the edge. This phenomenon, readily observable with the eye or in photographs of the sun, was examined by Abbott *et al.* (1922) at seven wave lengths between 3737 and 10,080 Å. Considering the intensity at the center of the disk to be unity for each wave length, it was found that the intensity decreased toward the edge of the sun for all wave lengths and that the diminution in intensity was more pronounced for short than for long wave lengths. For example, at a distance from the center equal to 0.95 of the photospheric radius, the intensity of wave length 3737 Å was 0.4319 of the central intensity, whereas at 10,080 Å it was 0.7331 of the central intensity. Thus the limb of the sun is not only less bright but is also redder than the center. The effect is explained by the considerations in the foregoing discussion and is probably due both to absorption and scattering by overlying layers of gases and to the fact

that radiations which emerge from the edge of the spherical sun come from higher and cooler levels than the radiations from the center of the disk which must pass through minimal thickness of overlying gases in escaping. Since the attenuation by scattering and absorption is greater for short than for long wave lengths, the effect is more pronounced in the blue than in the red end of the spectrum.

Although the sun is entirely gaseous, the sharpness of the photosphere, in comparison with more nebulous layers above it, has led to the custom of referring to these latter regions as the atmosphere of the sun. There are three regions of the solar atmosphere distinguishable by the states in

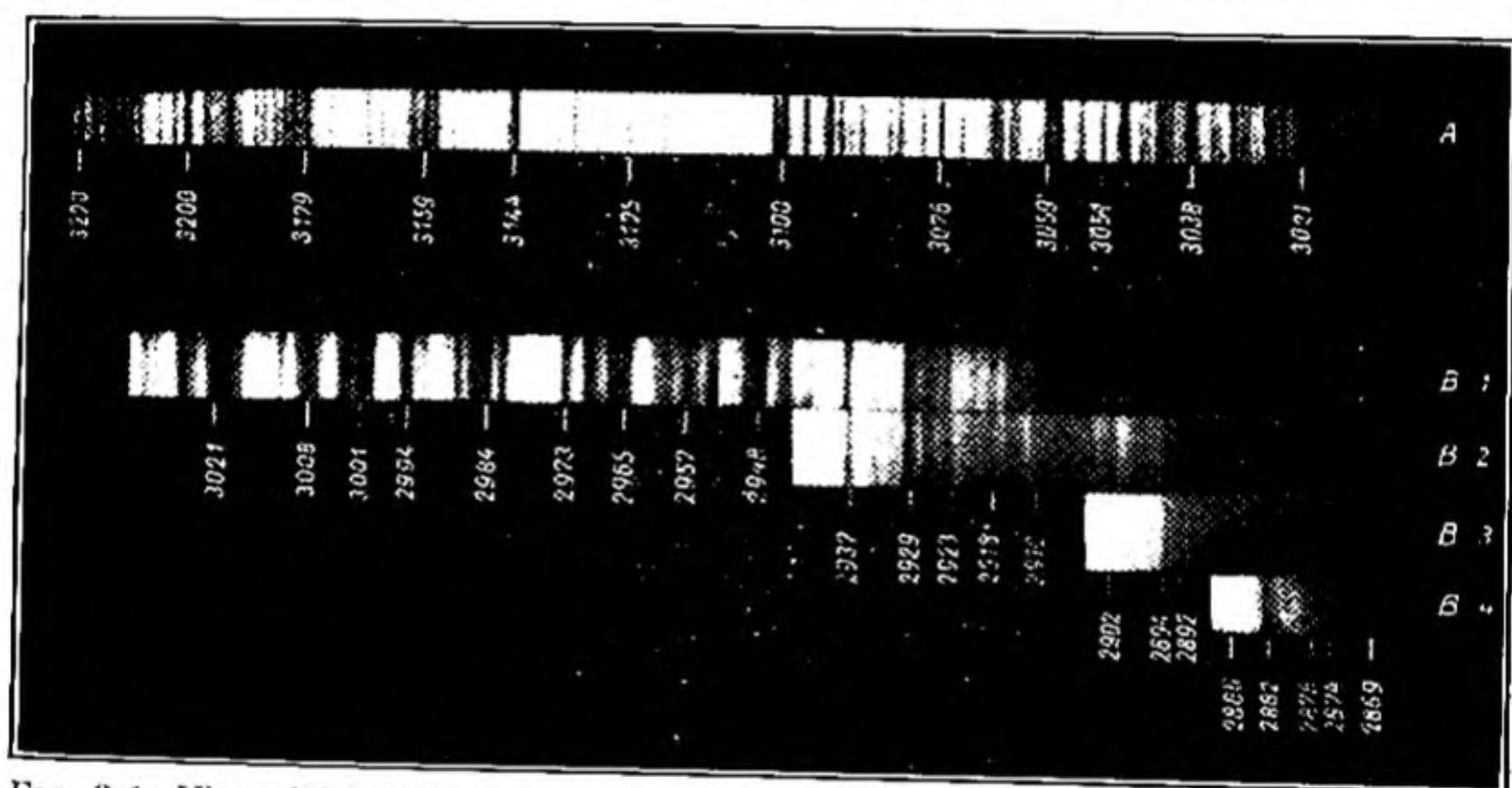


FIG. 3-1. Ultraviolet solar spectrum obtained in 1942 at Arosa, Switzerland. (Götz and Casparis, 1942.)

which matter exists in them and by the radiations which they emit—the reversing layer, the chromosphere, and the corona. The reversing layer is the innermost of these regions and lies just above the photosphere. It extends to a height of 1500 km above the photosphere and is the region in which the transition from continuous to line emission occurs. The temperature is about 4830°K, and the pressure is probably  $10^{-4}$  to  $10^{-3}$  atm. In the reversing layer the dark Fraunhofer lines of the solar spectrum are formed by atomic absorption at discrete wave lengths of the continuous radiation from the underlying hotter photosphere. The ultraviolet spectra of Figs. 3-1 and 2 show many of the large number of Fraunhofer lines in the biologically effective region of the solar spectrum. Babcock *et al.* (1948) have investigated the ultraviolet solar spectrum between 2935 and 3050 Å with a 21-ft grating spectrograph and have listed 665 absorption lines in this erythral region.

It must not be supposed that the dark lines represent points of zero intensity in the solar spectrum; they appear so in Figs. 3-1 and 2 because



of high photographic contrast. During a total eclipse of the sun when the moon obscures the photosphere, the reversing layer and regions above it are seen to emit brilliant spectral lines, the so-called "flash spectrum." Although the dark lines reduce the intensity of higher temperature radiation from the photosphere, they are only relatively dark and radiate toward the earth with intensities appropriate to a lower temperature source at about  $4800^{\circ}\text{K}$ .

The Fraunhofer absorption is stronger in the ultraviolet than in the visible solar spectrum. Pettit (1940) mapped the spectral energy between the Fraunhofer lines, using a high-dispersion spectrograph and

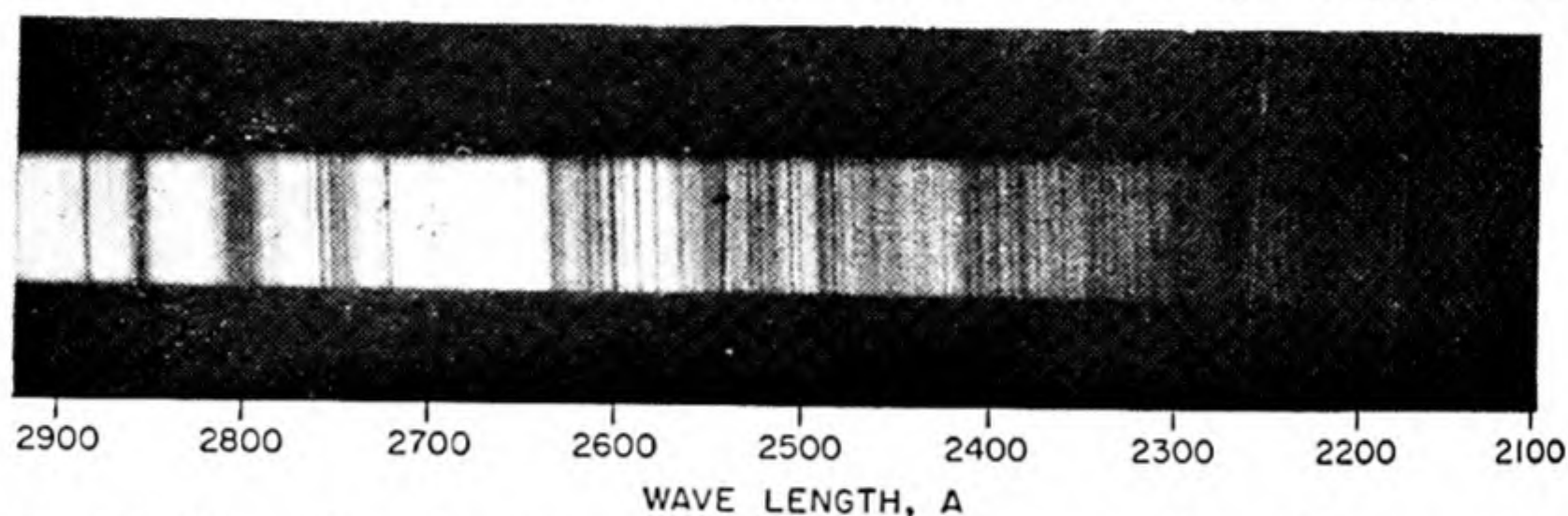


FIG. 3-2. Ultraviolet solar spectrum obtained during a rocket flight of June 14, 1949, by Johnson, Purcell, and Tousey (1952).

sensitive photocells, and, from his measurements, estimated the attenuation of radiation from the photosphere by the overlying layers of the sun. Comparison of his results for integrated radiation from the sun and for radiation between the Fraunhofer lines indicates that the total intensity between 3200 and 4000 Å is about 70 per cent of the radiation which would be emitted by the unobscured photosphere, whereas between 4000 and 7000 Å the ratio is about 91 per cent.

The chromosphere is a region consisting principally of hydrogen, helium, and calcium, located immediately above the reversing layer; it extends from the top of the reversing layer at about 1500 km above the photosphere to a height of 12,000 km which is the greatest height at which  $H_{\alpha}$ , the first Balmer line (6563 Å), occurs. Other lines of the Balmer series of hydrogen and line spectra of other un-ionized atoms fade out at lower heights. The flash spectrum of the chromosphere as seen during a solar eclipse contains emission lines of helium and ionized helium together with lines of ionized metals. Menzel points out the existence of ionized helium in the chromosphere and Hoyle gives results obtained from studies of line widths in the flash spectrum as evidence that the temperatures in this region are  $20,000\text{--}30,000^{\circ}\text{K}$ . Prominences originating in the chromosphere and sometimes rising several hundred thousand miles also contain matter at these temperatures. The chromosphere also emits continuous radiation, but none of these emissions—the line spectra, the spectra of

prominences, or the continuum—are strong. The quantity  $\alpha_{\lambda}h$  in Eq. (3-1) is small throughout the chromosphere, and light from the underlying layers is readily transmitted. Since the chromosphere is a weak absorber, it is a weak emitter.

The corona is the outermost observable region of the sun, being observable only during a solar eclipse or by use of the coronagraph. The corona begins in the region where the total continuous radiation from the solar atmosphere is about equal in intensity to the total line emission, and this region lies about 12,000 km above the photosphere. The corona extends outward for very great distances. S. P. Langley is said to have observed a coronal streamer extending to 12 solar diameters during the eclipse of 1878. Photographs usually show the corona extending to about 1 solar diameter because of the rapid decrease in its brightness with increasing height.

The visible light from the corona consists principally of light from the photosphere scattered by electrons, but emission lines of highly ionized calcium, iron, nickel, and argon are also present, the most intense being the green line at 5303 Å due to Fe(XIV), that is, iron with half its 26 electrons removed. Temperatures of about one million degrees are required to produce the states of ionization and other effects observed in the corona. Nevertheless, the entire visible light from the corona is about half that of the full moon, or about one one-millionth that of the sun, and its contribution to the visible light and ultraviolet radiations which reach the surface of the earth is inconsequential. The corona and upper chromosphere are, however, of interest as the source of radio waves and of X rays emitted by the sun. Although these emissions are probably too weak to be of biological importance, they deserve a brief description.

Radio emissions originating outside the earth, presumably from interstellar space, were discovered in 1932 by Jansky (1933) in experiments with 30-meter waves, but emissions from the sun were not known until 1945 when the improved sensitivity of receiving techniques brought about their detection (Hagen, 1951). It was found that the solar radio waves originate in the upper chromosphere and corona in thick regions of the solar atmosphere which center at heights above the photosphere of approximately 8000, 10,000, 13,000, and 18,000 km for wave lengths 0.8, 3, 10, and 50 cm, respectively. For example, during optical totality of the solar eclipse of May 20, 1946, the solar radiations in the respective wave lengths were reduced to 1, 6, 19, and 33 per cent of their values for the uneclipsed sun. To account for the observed radio-wave energy the temperatures of the regions which were emitting the wave lengths 0.8, 3, 10, and 50 cm were calculated to be 7000, 10,000, 26,000, and 212,000°K.

In contrast with the limb darkening of the sun for visible light, when observed with radio waves, the sun brightens at the limb, the brightening increasing with the wave length. The solar radio emission is not constant



in intensity but varies in an irregular and unpredictable manner. At the shortest wave length the intensity is most constant. The correlation with sunspot area is poor at the shortest wave length and improves at a wave length of about 10 cm. At longer wave lengths the fluctuations in the intensity become more erratic and abrupt. Vigorous investigation of all these phenomena is in progress.

X rays and short ultraviolet radiations from the sun which had never been detected at sea level were measured by narrow-band photon counters carried aloft in a rocket (Friedman *et al.*, 1951) on Sept. 29, 1949. Ultraviolet radiation in the wave-length band 1150–1350 Å was observed above 65 km altitude, and in the band 1425–1700 Å above 100 km. Solar X-ray emission was first recorded at 85 km with a counter sensitive from 0 to 10 Å, which indicated, because of the known absorption of the atmosphere, that the solar emission became undetectable below 7 Å. The measured intensities required effective temperatures of the emitting regions, again probably the upper chromosphere and corona, of 4500°, 5000°, and 10<sup>6</sup>°K for the bands 1425–1700, 1150–1350, and 7–10 Å, respectively. It seems nearly certain that X rays longer and softer than 10 Å are emitted by the sun, and further rocket experiments have been planned to investigate the subject.

#### SUNLIGHT ON TOP OF THE ATMOSPHERE

Two methods have been used to determine the solar spectral energy on top of the atmosphere: (1) by measuring the spectral intensity of the sunlight reaching the surface of the earth and correcting for the transmission of the terrestrial atmosphere, and (2) by sending apparatus on rockets to altitudes above most of the atmosphere. Both methods are difficult; the first has been in use for many years but can obviously give information about only those wave lengths which are detected at the earth's surface; the second is relatively recent and has yielded important new results in the ultraviolet portion of the spectrum. In the first method, unfocused radiation from the entire sun is allowed to fall on the slit of a double monochromator, that is, two monochromators in series in order to reduce contamination, by radiation scattered by the optical parts of the instrument, of the spectrum falling on the exit slit. Back of the exit slit, in a position to intercept the portion of the spectrum emerging from the monochromator is mounted a bolometer, a thermocouple, or a calibrated photoelectric cell with which the intensity may be measured point by point throughout the spectrum or in selected portions of it. Glass or quartz prisms and lenses in the monochromator are used to measure the major portion of sunlight reaching the surface of the earth, which lies between 2900 Å and 2.5  $\mu$ . Rock-salt prisms and diffraction gratings are used to observe the infrared solar spectrum from 2.5 to 25  $\mu$ . This portion of the spectrum has been of little direct interest in bio-

physics, although it is of importance in the physical state of the atmosphere and in meteorology.

The measurement of the spectral distribution of intensity in sunlight and its correction for atmospheric attenuation has been a major function of the Astrophysical Observatory of the Smithsonian Institution, beginning in 1892 under the direction of S. P. Langley who invented the bolometer and first measured the spectral distribution of energy in the solar spectrum, and continuing under Abbott and others. Their measurements have been made at Mt. Wilson, Mt. Whitney, and Washington, D.C. A convenient summary of their work is to be found in the Smithsonian Physical Tables (Fowle, 1934b). Many details of method are described in a later publication by Abbott *et al.* (1942). Independent and, in some spectral regions, improved measurements of solar spectral intensity have been made by other investigators. In nearly every case, however, the measurements were scaled to fit the Smithsonian curves which therefore remain the standards over most of the spectrum.

In the ultraviolet and visible portions of the spectrum it is observed that the atmospheric absorption follows an exponential law. Hence

$$i = i_0 e^{-\alpha \gamma \sec Z}, \quad (3-2)$$

where  $i$  and  $i_0$  = the intensities of a beam of sunlight at the bottom and top of the atmosphere, respectively, and  $\alpha$ , the atmospheric attenuation coefficient, refer to a wave-length interval from  $\lambda$  to  $\lambda + d\lambda$ ;  $Z$  = the zenith angle of the sun; and  $\gamma$  = a factor which accounts for the curvature of the earth. Values for  $\gamma$  are listed in standard tables;  $\gamma$  is very close to unity for  $Z < 80^\circ$ . In the infrared for certain bands of water vapor and other gases, Eq. (3-2) does not agree with the observed absorptions. However, Eq. (3-2) is not wrong; the discrepancy is due to the use of insufficient dispersion to resolve the narrow and complex structure of many of the bands.

The air mass  $M$  is defined by

$$M = \gamma \sec Z. \quad (3-3)$$

From Eq. (3-3),  $M = 1$  for  $Z = 0$  and  $\gamma = 1$ , and therefore  $M$  is the amount of atmosphere from the surface to space in a vertical direction. Then

$$i = i_0 e^{-\alpha M}. \quad (3-4)$$

To determine  $i_0$  on top of the atmosphere,  $i$  is measured for several values of  $M$  and is plotted for each wave length on a logarithmic scale against  $M$ . The straight line thus obtained is extrapolated to zero air mass, which gives  $i_0$  when proper account is taken of the transmission of the spectrograph and the spectral response of the accompanying bolome-



ter or photoelectric cell. The determination requires that the atmospheric attenuation  $\alpha$  remain unchanged for several hours as  $Z$  changes.

Several sets of data obtained by this method are plotted in Fig. 3-3. They include the data of the Smithsonian Institution (Abbott *et al.*, 1923; Fowle, 1934b) of Pettit (1940) at Mt. Wilson, and of Götz and Schönmann (1948) at Arosa. The curve of Moon (1940) is an average of all data existing in 1940. The data at an altitude of 55 km were obtained in 1947 by Durand *et al.* (1949) of the Naval Research Laboratory with a spectrograph on a rocket; at 55 km the pressure was  $2 \times 10^{-4}$

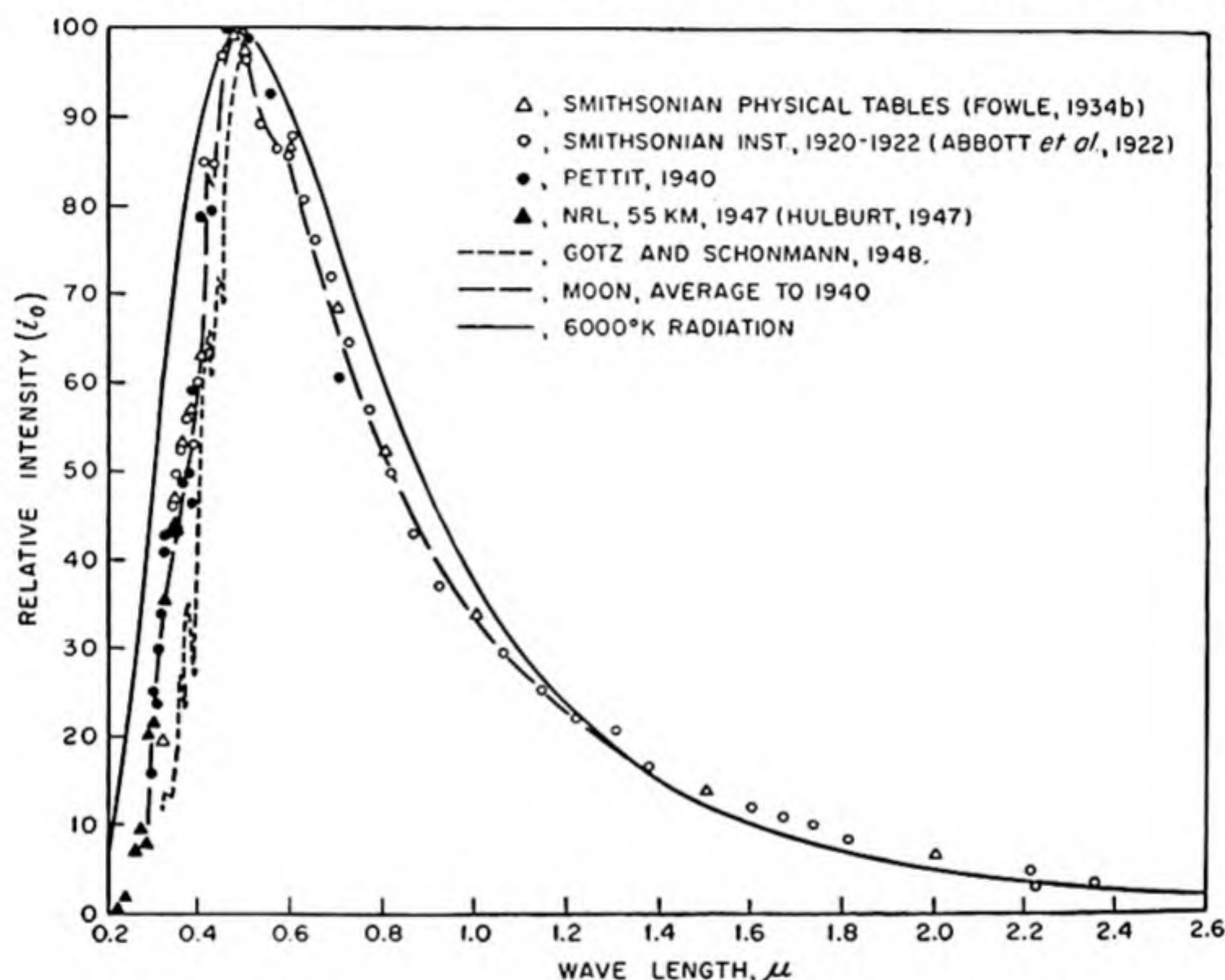


FIG. 3-3. Solar-spectrum curves on top of the atmosphere.

atm, and the spectrograph had risen through 4999/5000 of the atmosphere and had only 1/5000 above it. The solid-line curve of Fig. 3-3 is the spectral intensity of a black body at 6000°K. All the curves of Fig. 3-3 were arbitrarily adjusted to have their maxima at 100. It is seen that the 6000°K curve lies above the solar values in the ultraviolet and also in the red and infrared to 1.4  $\mu$ .

In Figs. 3-1 and 2 are reproduced perhaps the best photographs which have been made of the ultraviolet portions of the solar spectrum. The spectrum of Fig. 3-1 was taken by Götz and Casparis (1942) at Arosa, Switzerland, using all possible care to reduce the scattered light which always veils the short-wave-length limit of the solar spectrum below the ozone layer. The spectrum of Fig. 3-2 is a composite made from four of a series of spectra obtained by F. S. Johnson, J. D. Purcell, and R.

Tousey (1952) of the Naval Research Laboratory with a spectrograph on a rocket flying above the ozone region from about 60 to 110 km on June 14, 1949. The spectra of Figs. 3-1 and 2 overlap at the absorption band 2882 Å.

The curves of Pettit and the NRL (Durand and coworkers, unpublished; cited in Hulburt, 1947) shown in Fig. 3-3 in the ultraviolet, are replotted in Fig. 3-4 with the ordinate on an absolute scale and with the

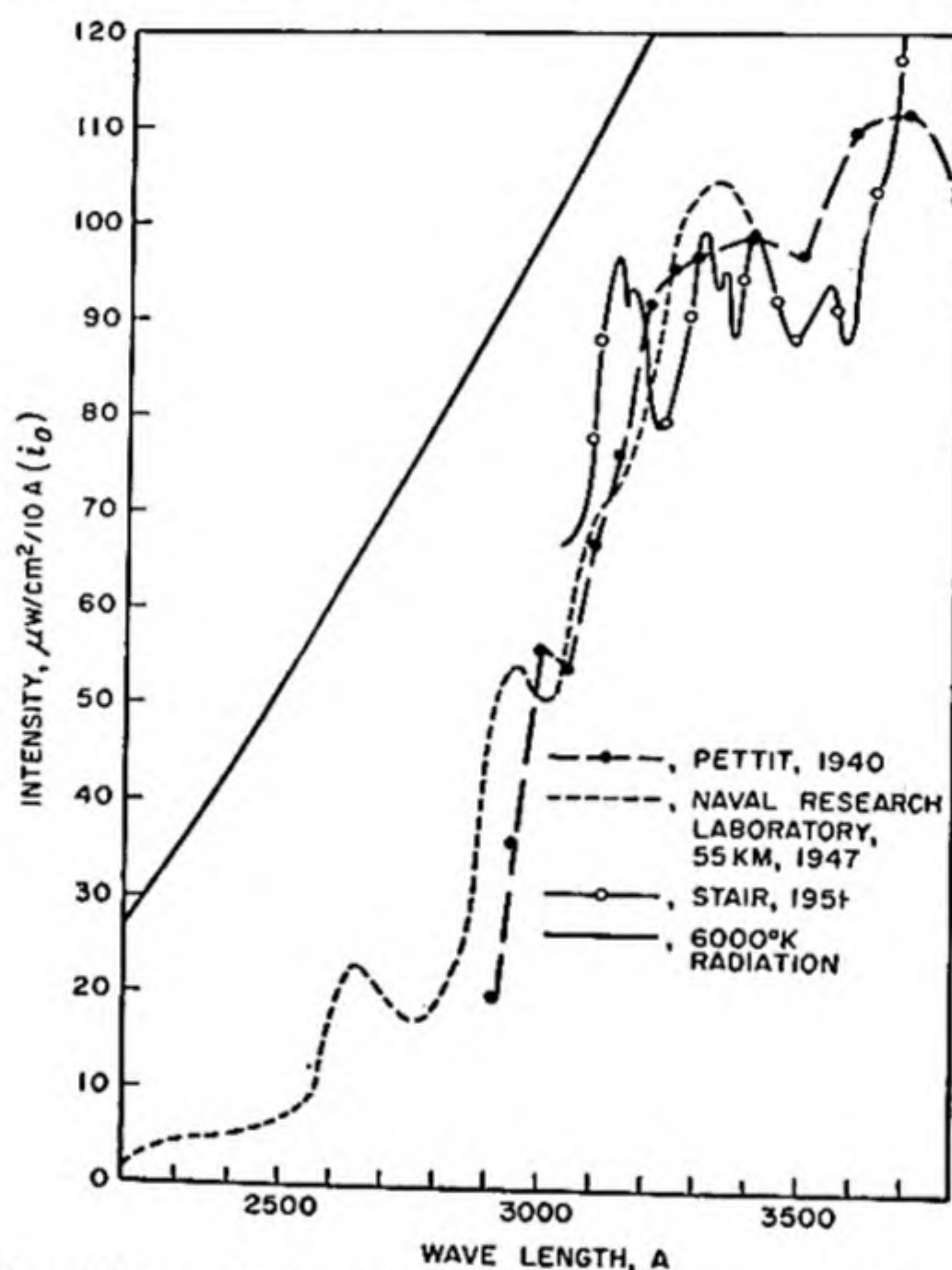


FIG. 3-4. Ultraviolet portion of the solar spectrum on top of the atmosphere.

scale of the abscissa expanded over that of Fig. 3-3 in order to bring out detail. The data of Stair (1951) of October 1950 are added. The fluctuations in the curves indicate certain major absorptions in the solar spectrum but are not fine-grained enough to bring out all the Fraunhofer lines. Pettit's value of  $i_0$  at 3400 Å is taken as standard, and the other two curves are adjusted to it. The 6000°K black-body curve of Fig. 3-3 is continued in Fig. 3-4 to emphasize the fact that the solar intensity continues to fall below it as the wave length is decreased to 2200 Å.

In the infrared beyond 2.6  $\mu$ ,  $i_0$  decreases rapidly with increasing wave length approximately as for a black body at 7000°K. For example, rela-



tive to the maximum intensity at 4600 Å the intensities at 5, 8, and 14  $\mu$  are  $1.9 \times 10^{-3}$ ,  $3 \times 10^{-4}$ , and  $3.7 \times 10^{-5}$ , respectively, but the idea that  $i_0$  is approximate to or is less than the intensity of a black body at 6000°K must not be pushed too far because, as brought out in the preceding section, both in the region of X rays and of radio waves the solar energy is such as to indicate the existence of emissive regions of the sun, probably in the corona, which are at temperatures much greater than 6000°K.

In Table 3-1 is given the energy in several portions of the solar spectrum on top of the atmosphere calculated from the areas under the average of the curves of Figs. 3-3 and 4. In obtaining the values in column

TABLE 3-1. SPECTRAL DISTRIBUTION OF SOLAR RADIATION

Wave-length interval	Fraction of total radiation	Flux density cal/cm <sup>2</sup> min
2200–3150 Å	0.014	0.027
3150–4000 Å	0.079	0.153
4000–7000 Å	0.403	0.78
7000 Å–2.6 $\mu$	0.474	0.92
2.6–14 $\mu$	0.03	0.06

3 of the table the solar constant, which is the flux density of total solar radiation on top of the atmosphere at the earth's mean distance from the sun, was taken to be 1.94 cal/cm<sup>2</sup> sec. It is seen from Table 3-1 that about half the total solar radiation lies in the visible and ultraviolet regions below 7000 Å and about half in the infrared.

### SOLAR ULTRAVIOLET RADIATION AT THE EARTH'S SURFACE

The intensity of the solar ultraviolet radiation that reaches a particular point on the surface of the earth depends on the amount of ozone, air haze, and clouds between that point and the sun. Of these materials, ozone is important because of the absorption of its great ultraviolet band which begins at about 3400 Å and increases rapidly for shorter wave lengths. Air, haze, and clouds attenuate the rays of the sun mainly by scattering with little true absorption; the scattering, of course, causes the sky. For ultraviolet radiation, true absorption of the oxygen of the air does not set in appreciably until the wave length becomes less than about 2700 Å.

The absorption coefficient  $\alpha$  of ozone, observed by Tsi-Ze and Shin-Piaw (1932, 1933) is plotted in curve I, Fig. 3-5, against the wave length; about one-fifth the observations are shown by the dots. The absorption coefficient  $\alpha$  is defined by

$$i = i_0 e^{-\alpha x}, \quad (3-5)$$

where  $i_0$  and  $i$  are the intensities of a collimated beam of light in the wave-length region from  $\lambda$  to  $\lambda + d\lambda$ , entering and emerging from a layer of ozone  $x$  cm in thickness at normal temperature and pressure (NTP), i.e., at  $0^\circ\text{C}$  and a pressure of 1 atm. The long-wave-length region of the ozone absorption from about 3400 to 3100 Å has a band structure known as the Huggins bands; the smooth absorption below 3100 Å is

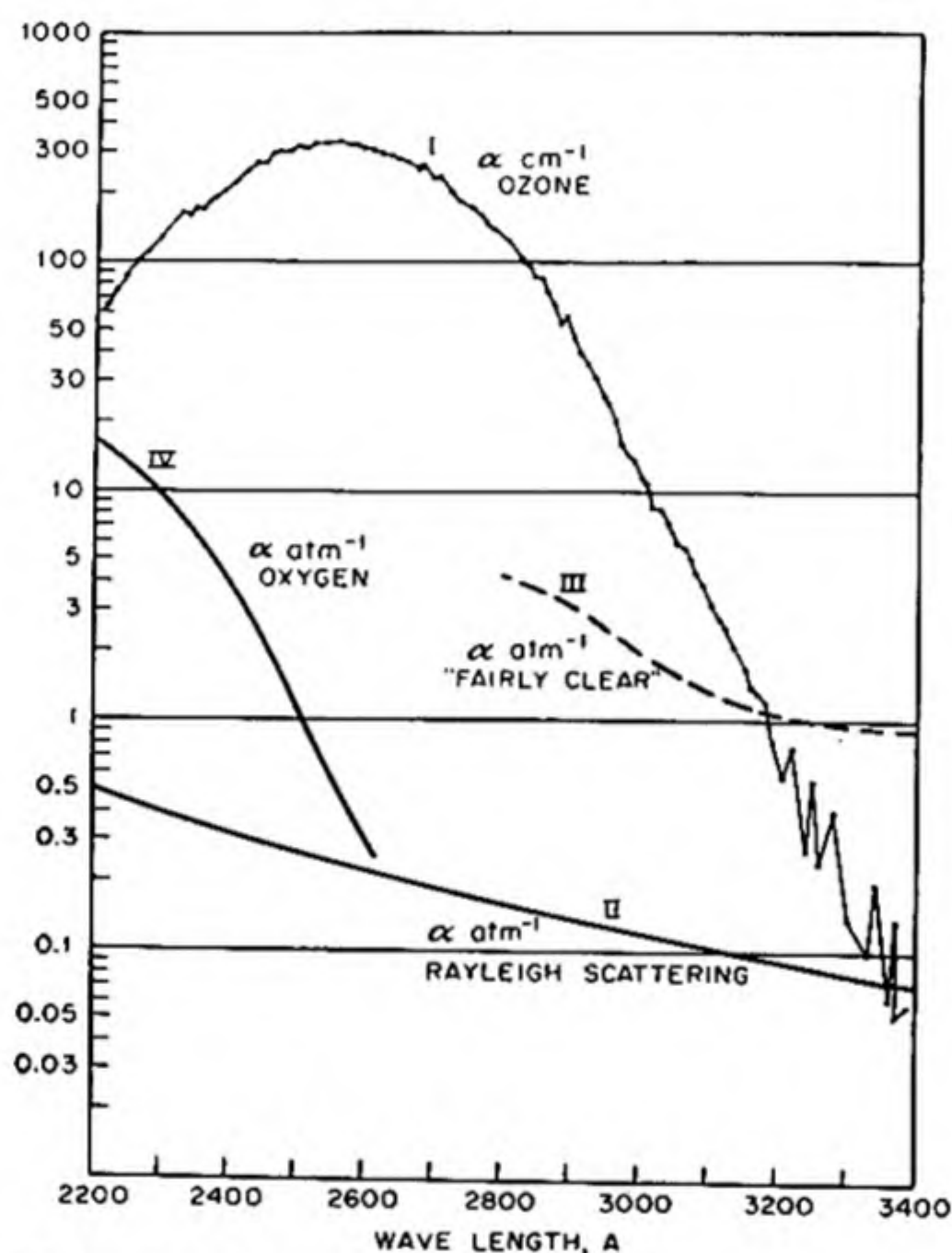


FIG. 3-5. Ultraviolet absorption coefficients of some atmospheric gases.

known as the Hartley continuum. The absorption in the Huggins bands varies with the temperature in a complicated way, which is of no interest here; roughly,  $\alpha$  decreases between 10 and 30 per cent when the temperature falls from  $18^\circ$  to  $-50^\circ\text{C}$  (Vigroux, 1950). It is seen from the ozone curve of Fig. 3-5 that, below 3100 Å,  $\alpha$  rises to very high values; therefore it is not surprising that even the few millimeters of ozone which exists in the upper atmosphere is sufficient to prevent the detection of ultraviolet wave lengths from 2915 to 2150 Å at the surface of the earth.

In the standard atmosphere the pressure is  $1.0132 \times 10^6$  dynes/cm<sup>2</sup>, and the total air in a vertical column of the standard atmosphere from sea level to space amounts to 8 km of air at NTP; 20.75 per cent of this is oxygen. In Fig. 3-5, curve II is the attenuation coefficient  $\alpha$  per atmos-



phere of pure air calculated from the Rayleigh scattering theory with polarization defect, and curve III is  $\alpha$  per atmosphere observed by Vassy (1941) for what may be termed a "fairly clear atmosphere at sea level." The observed (Buisson *et al.*, 1930, 1932) absorption coefficient  $\alpha$  of the molecular oxygen gas contained in 1 atm, from which the Rayleigh term has been subtracted, is plotted in curve IV, Fig. 3-5. There are no available data on the attenuation of ultraviolet radiation passing down through a hazy or cloudy atmosphere.

### ATMOSPHERIC OZONE

Ozone exists in the upper atmosphere of the earth for the most part in the region between 15 and 35 km above sea level; it extends in rapidly

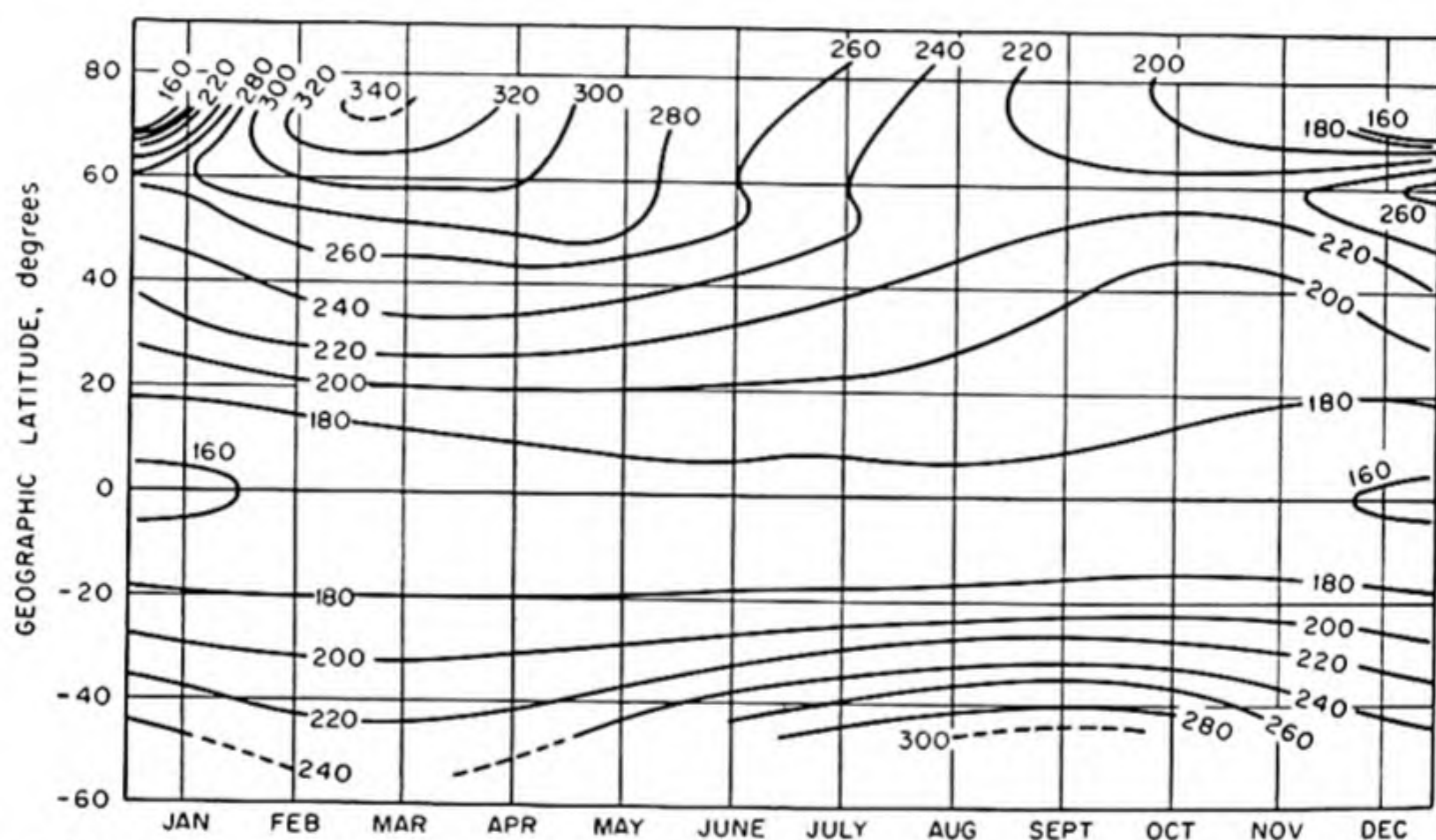


FIG. 3-6. Average contours of equal thickness of ozone. Unit,  $10^{-3}$  cm of ozone at NTP. (Götz, 1944.)

decreasing concentration as high as 50 km and down to sea level where there are often traces amounting to as much as 0.05 mm of ozone at NTP per kilometer of air (or  $5 \times 10^{-7}$  by volume of air). The total thickness of ozone in a vertical column of air from sea level to space varies from about 1.5 to 5 mm at NTP depending on the latitude and the season. This is brought out in Fig. 3-6 which gives an average world-wide distribution of ozone derived by Götz (1944) from a summary of nearly all available observations. Figure 3-6 shows the increase of ozone thickness with increasing latitude up to about  $60^\circ$  for all seasons of the year and the spring maximum and autumn minimum for latitudes greater than  $20^\circ$ .

Whether there is a diurnal variation in the ozone thickness above any station is not known with certainty; a few data mentioned by Dobson

(1930) indicate that a diurnal variation, if it exists, is small. Regarding the change of ozone thickness with sunspots, there are few long-continued series of ozone data. Fowle (1934a, 1935) obtained yearly average values at north latitude  $34^\circ$  in Arizona and California. His values from 1921 to 1928 varied with the sunspots, but the correspondence was not maintained from 1928 to 1934. A connection between ozone and sea-level barometer changes is complicated and has not been clearly established. From the data of six ozone stations in Europe, Dobson concluded that the smallest amount of ozone occurred to the southwest of a barometric high and that the passage of a barometric low over a station was accompanied by an increase in ozone content. In general, since most of the ozone lies between 15 and 35 km, its variations would be expected to be correlated with changes of pressure and winds in the stratosphere rather than in the troposphere, and at this time the relation between stratospheric and tropospheric weather cannot be said to be completely known and understood.

#### CALCULATED ULTRAVIOLET INTENSITY AT THE EARTH'S SURFACE

A complete calculation of the solar ultraviolet radiation falling on the surface of the earth from the sun and sky is very complicated and will not be attempted. It is, however, instructive to calculate the ultraviolet intensity of the direct rays of the sun at the surface of the earth for various zenith angles of the sun. Let  $i_0$  and  $i$  be the intensities, respectively, of the rays of the sun outside the atmosphere and at the surface of the earth. Then

$$i = i_0 e^{-(\alpha x + \alpha_a) \gamma \sec Z}, \quad (3-6)$$

where  $\alpha$  = the absorption coefficient of ozone per centimeter,  
 $x$  = the thickness of the ozone in centimeters at NTP,  
 $\alpha_a$  = the attenuation per atmosphere below the ozone region due to pure air and haze, and  
 $Z$  = the zenith angle of the sun.

The symbols  $i$ ,  $i_0$ ,  $\alpha$ , and  $\alpha_a$  refer to the wave-length region from  $\lambda$  to  $\lambda + d\lambda$ . In Fig. 3-7 the curve labeled  $i_0$  is the Naval Research Laboratory curve of Fig. 3-4 and is plotted in arbitrary units against the wave length for the ultraviolet region of the solar spectrum where the absorption of ozone becomes important. The values of  $i$  for ozone of thickness 4 mm, for no atmospheric attenuation, and for various zenith angles were calculated from Eq. (3-6) with  $x = 0.4$  cm,  $\alpha_a = 0$ , and values of  $\alpha$  obtained by drawing a smooth curve through the fluctuations of curve I, Fig. 3-5. The  $i$  vs.  $\lambda$  curves are plotted in Fig. 3-7 for six values of  $Z$ . The total ultraviolet energy  $E$  in wave lengths less than 3200 Å was



calculated from the integral

$$E = \int_0^{3200} i \, d\lambda \quad (3-7)$$

and the  $i$  vs.  $\lambda$  curves for the several values of  $Z$ . The resulting values of  $E$  are plotted against  $Z$  in the 4-mm curve of Fig. 3-8. Families of  $i$  vs.  $\lambda$  curves similar to those of Fig. 3-7 were calculated for other ozone thicknesses, and from these curves and Eq. (3-7) the  $E$  vs.  $Z$  curves of

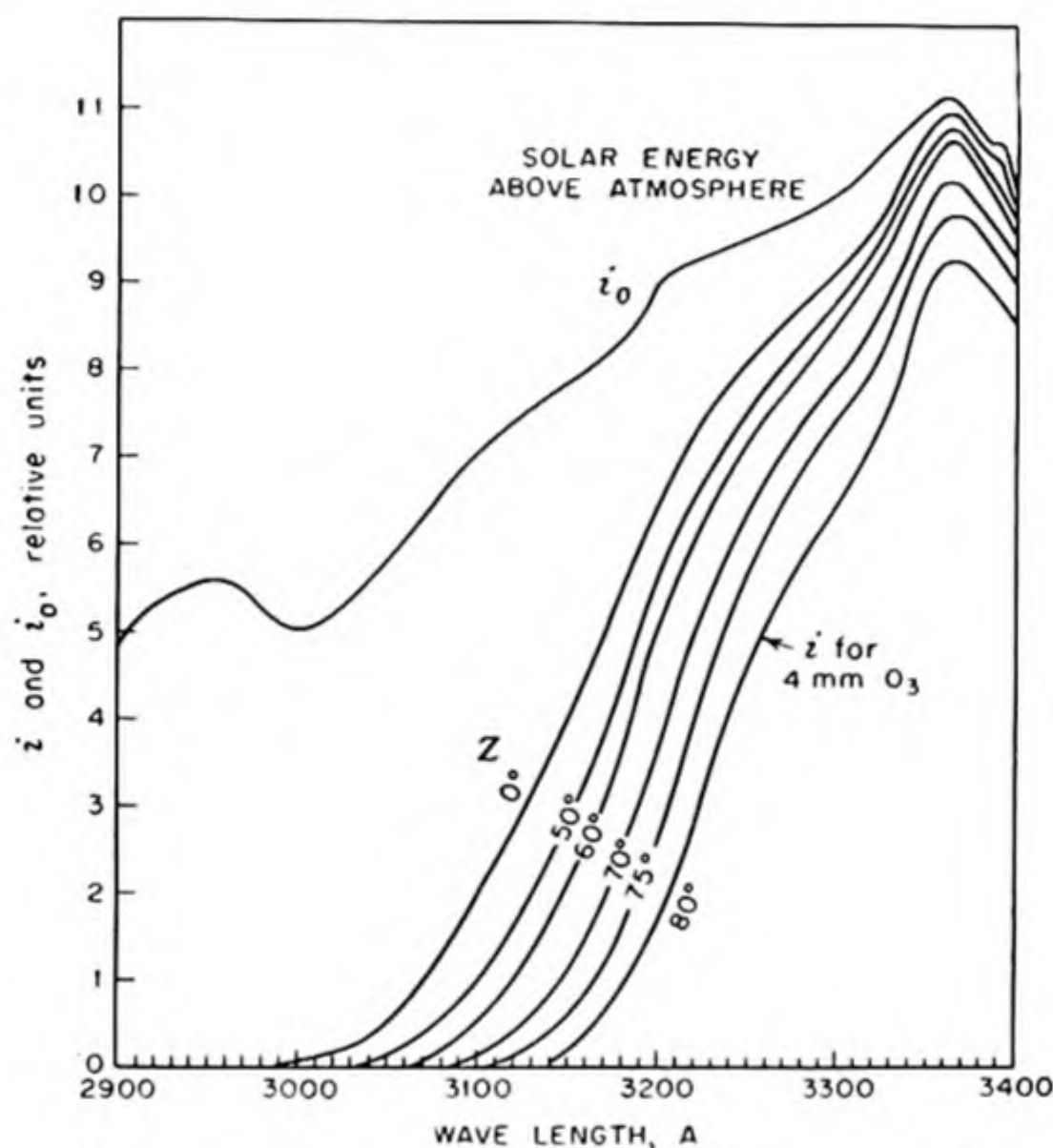


FIG. 3-7. Observed solar-spectral-energy curve  $i_0$  above atmosphere and calculated curves of  $i$  after passing through 4 cm of ozone at various zenith angles.

Fig. 3-8 for 1, 2, and 3 mm of ozone were calculated. The curves illustrate the manner in which  $E$  decreases with increasing ozone thickness and increasing zenith angle. Curves for integrals to wave lengths less than 3200 Å were similar to those of Fig. 3-7 but descended more rapidly to low values.

The effect of the atmosphere, in addition to the ozone, is shown by the dotted curve of Fig. 3-8, which refers to a "fairly clear" atmosphere with 1 mm of ozone. It was calculated from Eqs. (3-6) and (3-7) with  $\alpha_a$  from curve III, Fig. 3-5. The dotted curve of Fig. 3-8 brings out the almost obvious fact that a relatively small amount of atmospheric haze is more effective in reducing the ultraviolet energy of the direct rays of the sun than, for example, doubling the ozone thickness of the upper atmosphere. However, we must hasten to remark that haze reduces the total ultravio-

let radiation falling on the earth's surface far less than it reduces the effectiveness of the direct rays of the sun. The reason is that the light scattered out of the direct solar rays by haze is not entirely lost, since haze exerts little absorption, but reappears as sky light, which for thin haze is mainly directed downward.

A fraction of the sky light is scattered outward to space and is lost to the earth; the fraction increases with increasing haze and clouds. As has been mentioned, an exact calculation of all this is complicated and would require a complete theory of sky brightness and polarization for ultraviolet wave lengths in terms of the ultraviolet optical constants of the atmosphere in all stages of haziness. Such a theory has not been formulated, and

such constants have not been determined; therefore only a survey of the observational material is presented in the following section.

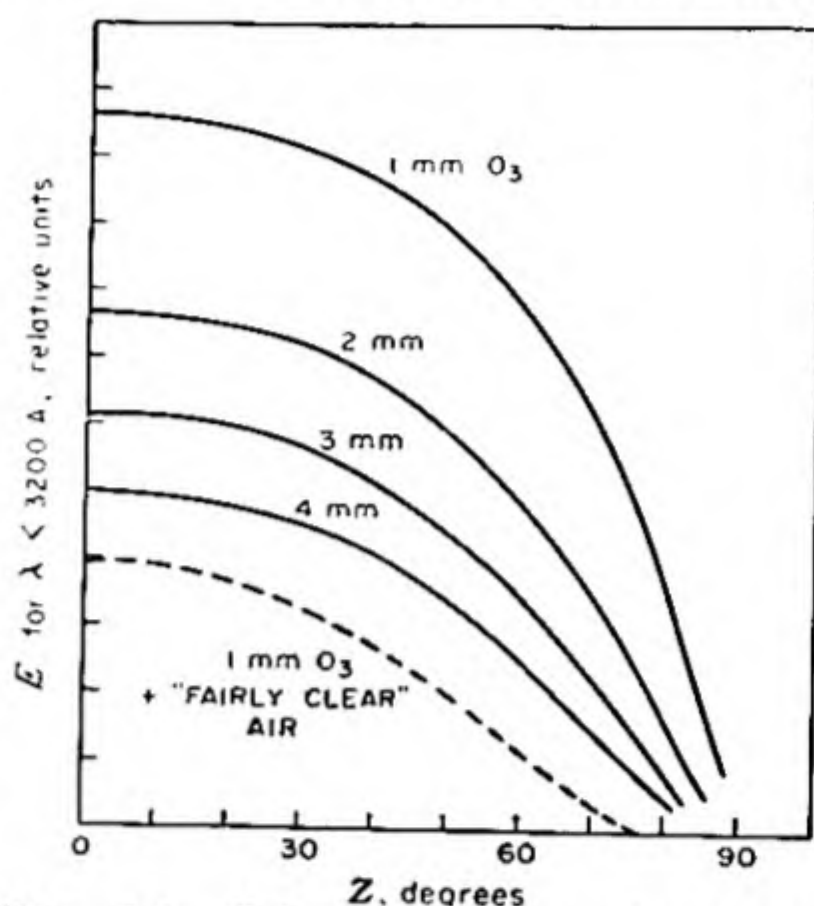


FIG. 3-8. Calculated solar ultraviolet energy  $E$  for wave lengths less than 3200 Å for several cases.

### OBSERVED ULTRAVIOLET INTENSITY AT THE EARTH'S SURFACE

Many measurements of ultraviolet radiation from the sun and sky were carried out at Washington, D.C., by Coblentz and Stair (1943). The first series of measurements were made with photocells arranged to record  $E_n$ , the radiation received on a plane normal to the rays of the sun, from the sun and a circular region of sky around the sun as a center  $22^\circ$  in diameter. Data were taken during the clearest days over the years 1936 to 1941; for illustration the values of  $E_n$  for 1937 are shown in Fig. 3-9. Two types of photocells were used, one sensitive to wave lengths less than 3200 Å and the other sensitive to wave lengths less than 3132 Å; the spectral-sensitivity curves of the photocells were not reported. In Fig. 3-9 the abscissas are the air mass  $M$  and the zenith angle of the sun  $Z$ , and the two scales of ordinates are the  $E_n$  in microwatts per square centimeter for the respective types of photocells. It is seen that they were approximately proportional and that the ultraviolet intensities of wave lengths below 3200 Å were roughly 2.5 times the intensities below 3132 Å. The data of the other years from 1936–1941 were similar to those of Fig. 3-9 for 1937. For any zenith angle the variation of the ultraviolet intensity of a factor of about 2, shown in Fig. 3-7, was proba-



bly due largely to haze variations within the qualitative specification of a "clear" day and, to a lesser extent, to ozone variations. No ultraviolet variation with sunspots appeared, and if any existed, it was obscured by the haze variations. The sunspot numbers for the years 1936-1941 were 80, 114, 110, 89, 68, and 48, respectively. There was an ill-defined seasonal variation in  $E_n$ , which is not brought out in Fig. 3-9, partially attributable to the spring-to-fall decrease in ozone (see Fig. 3-6), in that the ultraviolet intensity was often greater in the autumn than in the spring from equal solar zenith angles. However, high  $E_n$  values frequently occurred in late winter and early spring which may have been

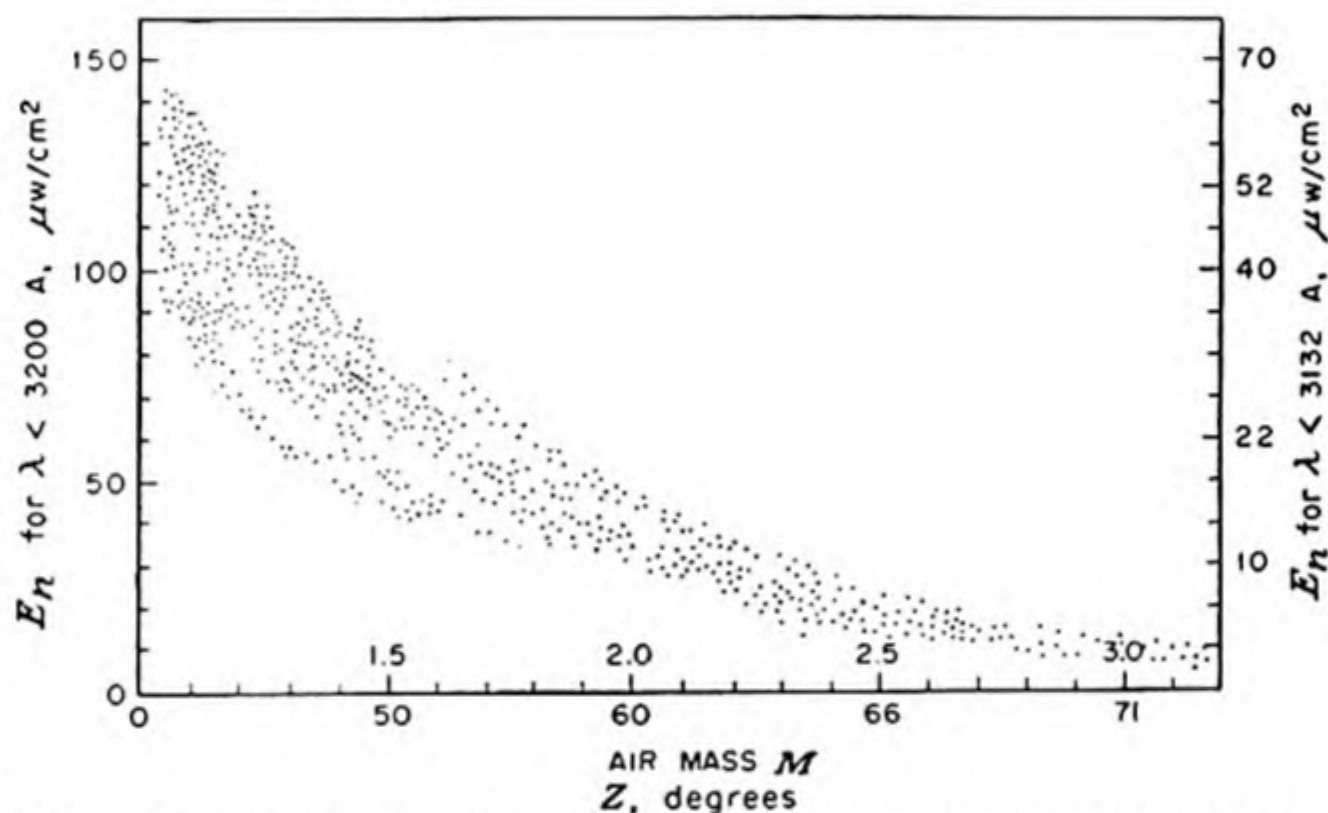


FIG. 3-9. Ultraviolet intensity  $E_n$  during clear days in Washington, 1937. (Coblentz and Stair, 1943.)

due to unusually clear skies or to local ozone variations attributable to stratospheric weather.

After 1941 Coblentz and Stair (1944) changed their plan of observation and measured  $E_h$ , the ultraviolet energy from the sun and the entire hemisphere of the sky, falling on a horizontal plane, instead of  $E_n$ , the ultraviolet energy from the sun and  $22^\circ$  of the sky, falling on a plane normal to the sunbeams. Values of  $E_h$  obtained for clear days in Washington are plotted in Fig. 3-10 as dots, circles, crosses, and triangles for June 4, Sept. 18, and Dec. 21, 1943, and Oct. 17, 1944, respectively. In Fig. 3-10 the two dotted curves outline the spread of value of  $E_n$  for clear days in Washington during 1936 and 1941. Since the points fell in the region between the dotted curves, it was concluded that  $E_h$  and  $E_n$  were approximately the same; in general, of course,  $E_h$  and  $E_n$  cannot be expected to be equal. Values of  $E_h$  for some clear days in high latitudes, obtained (Coblentz *et al.*, 1942) on a trip to Greenland in 1941 are shown by the solid lines of Fig. 3-10. The increase in  $E_h$  with latitude may have been due to less haze and less ozone at the higher latitudes, but these features were not measured.

A series of measurements of  $E_h$  for ultraviolet wave lengths less than

3022 Å was made in Cleveland in 1936 and 1937 by Luckiesh *et al.* (1937, 1939). The results exhibited about the same variation with  $Z$  and the same spread as the Washington data of Fig. 3-9 and therefore are not plotted in Fig. 3-10. For wave lengths less than 3022 Å,  $E_h$  was about one-fourth the  $E_h$  for wave lengths less than 3200 Å.

For ultraviolet wave lengths less than 3130 Å,  $E_h$  was observed during 1932 and 1933 by Ives and Gill (1937) in 14 cities scattered over the United States. In Fig. 3-11 their results are given for two groups, where group I refers to the most smoky localities and group III refers to the

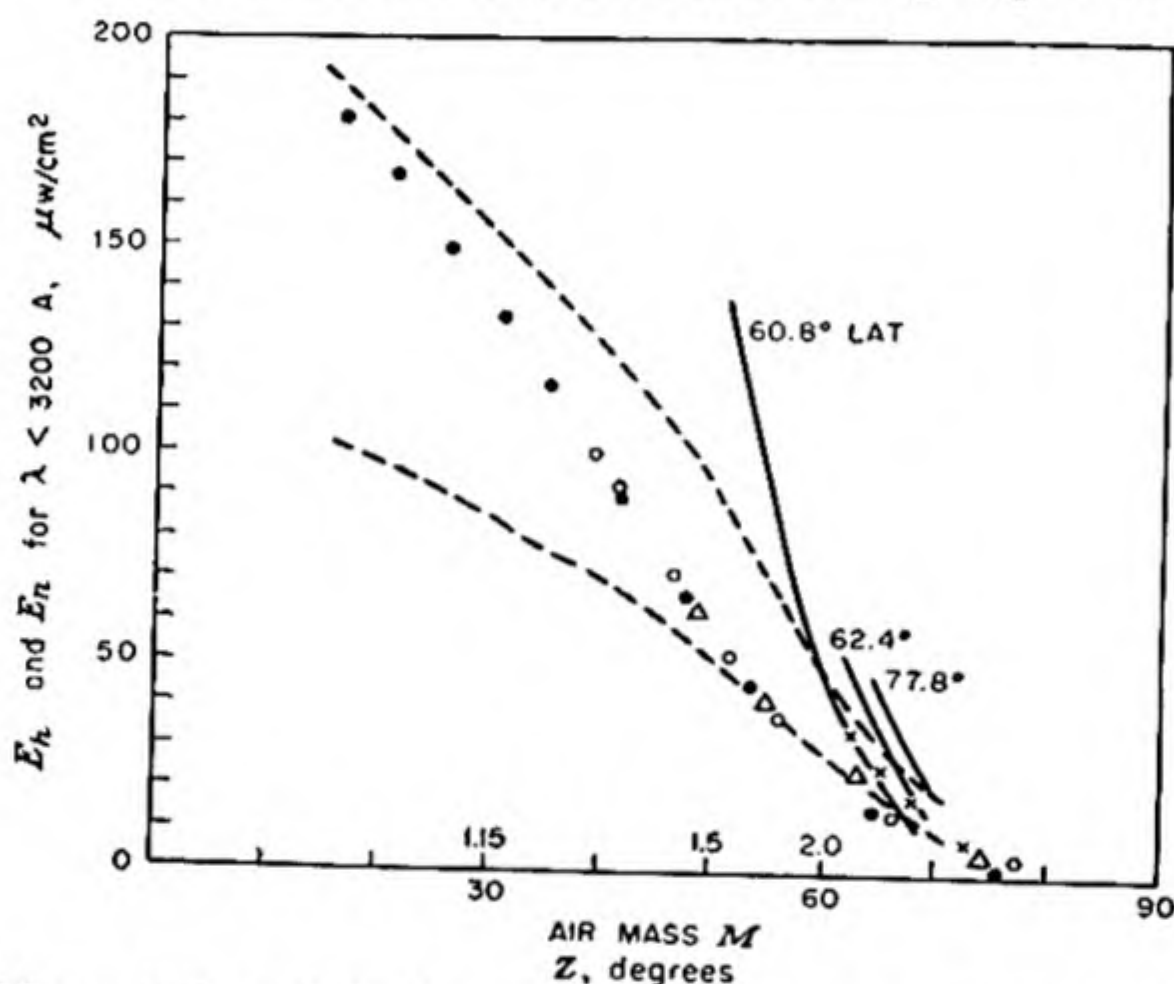


FIG. 3-10. Values of  $E_h$  and  $E_n$  for wave lengths less than 3200 Å. The symbols indicate  $E_h$  for some clear days in Washington during 1942 and 1943. The solid-line curve is for  $E_h$  for higher latitudes in 1941. The  $E_n$  for clear days in Washington 1936–1941 was within the dotted curves. (Coblentz and Stair, 1944; (Coblentz *et al.*, 1942.)

least smoky localities; the data for clear and cloudy skies were plotted separately. It is seen in Fig. 3-11 that the curves for the more smoky localities lie below those for the less smoky both for clear and cloudy skies and also that the curves for cloudy skies lie below those for clear skies. Therefore both smoke and clouds decreased the amount of ultraviolet radiation that reached the surface of the earth. Comparison of Fig. 3-10 with the "clear-sky" data of Fig. 3-11 shows good agreement, when it is remembered that  $E_h$  for wave lengths less than 3200 Å is about 2.5 times  $E_h$  for wave lengths less than 3130 Å.

The conclusion is therefore that, from Fig. 3-10, a rough estimate may be made, correct perhaps within a factor of 2, of  $E_h$  in clear weather for all seasons of the year and all times of the day. If a more exact value of  $E_h$  is required, provision must be made to measure it. To make the rough estimate, an average curve was drawn through the data of Fig.



3-10 which gave  $E_h$  in terms of the zenith angle of the sun. From this,  $E_h$  was calculated throughout the day for various latitudes and seasons. The results are plotted in Fig. 3-12 for the twenty-second day of March, June, September, and December for north latitudes  $0^\circ$ ,  $20^\circ$ ,  $40^\circ$ ,  $60^\circ$ , and  $80^\circ$ . The curves for March and September are the same at all latitudes because the zenith angle of the sun is the same at these epochs; at the

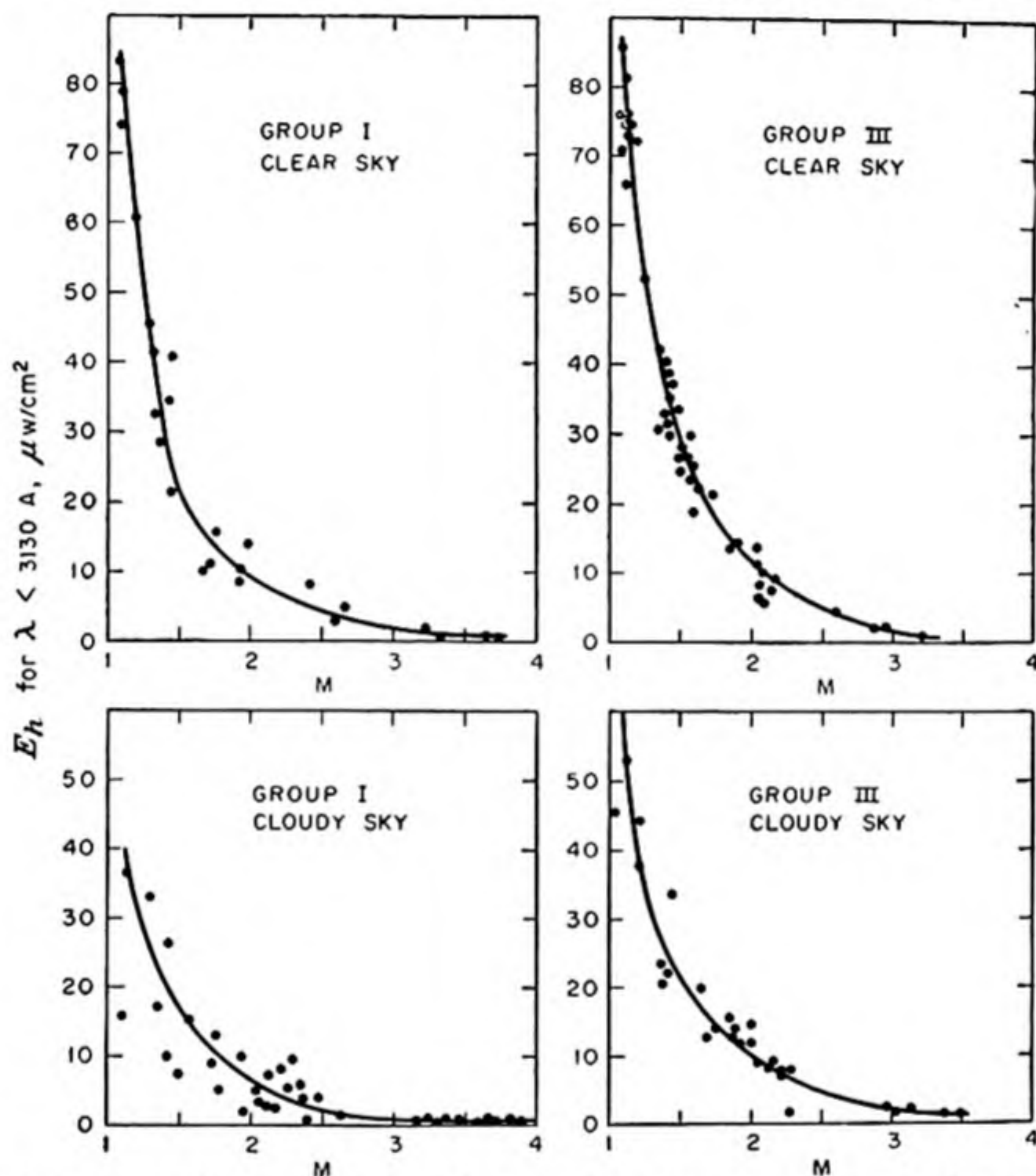


FIG. 3-11. Values of  $E_h$  for wave lengths less than 3130  $\text{\AA}$ . Group I, for most smoky localities; Group III, for least smoky localities. (Ives and Gill, 1937.)

equator the curves for June and December are the same for the same reason. For December at  $60^\circ$  north latitude and for March, September, and December at  $80^\circ$ , the sun does not rise above  $10^\circ$  above the horizon, and  $E_h$  is zero throughout. At  $80^\circ$  the value of  $E_h$  in June remains above zero all night, being 3 at midnight because the midnight sun is  $14^\circ$  above the horizon. In the curves of Fig. 3-12, no seasonal adjustments have been made for the fact that the sun is about 3 per cent nearer the earth on December 22 than it is on June 22. Since the values of  $E_h$  of Fig. 3-12 are based on the data of Fig. 3-10 obtained on clear days in the United States, they may be expected to be correct for localities outside the United States only if the localities have the same ozone and the same

clearness of atmosphere and sky as those that occurred in the United States.

A few measurements have been made by Luckiesh *et al.* (1939) of the ratio of the ultraviolet radiation on a horizontal plane from the total

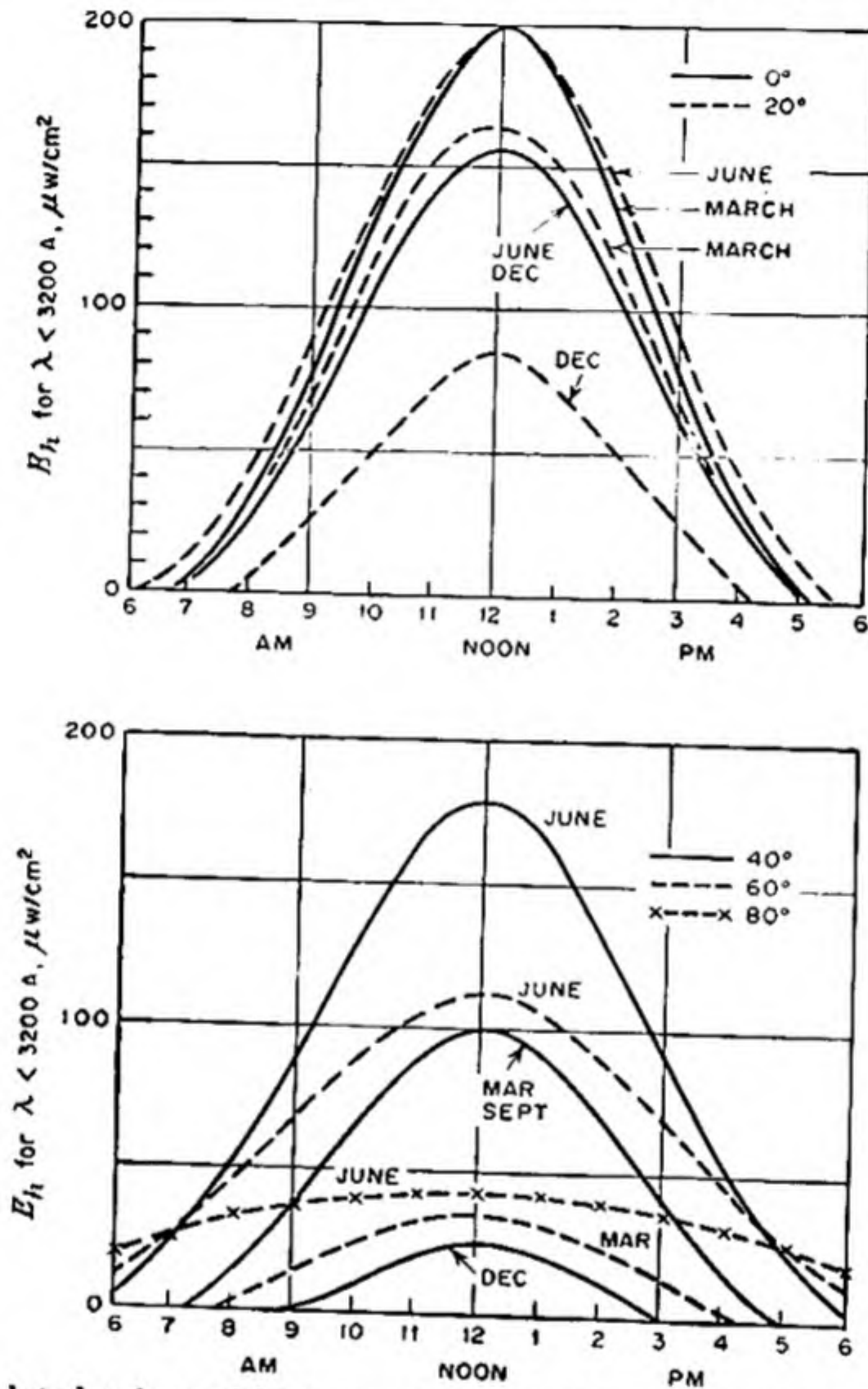


FIG. 3-12. Calculated values of  $E_h$  for wave lengths less than 3200  $\text{\AA}$  through the day for various seasons and latitudes.

hemisphere of the sky to that from the sun, i.e.,  $E_h(\text{sky})/E_h(\text{sun})$ . For clear days in Cleveland in 1936 and 1937 for wave lengths less than 3022  $\text{\AA}$  the ratio was 1.0, 1.1, 1.5, 2.2, 4, and 10 for zenith angles  $20^\circ$ ,  $30^\circ$ ,  $40^\circ$ ,  $50^\circ$ ,  $60^\circ$ , and  $70^\circ$ , respectively. During the mid-day hours of June 13, 1928, a very clear day, for zenith angles about  $20^\circ$ , Pettit (1932) found that, for wave lengths less than 3200  $\text{\AA}$ , the ratio was 1, 0, 0.55, and 0.43 at altitudes above sea level in the vicinity of Pasadena, Calif., of 845, 3400, and 5700 ft, respectively. In general, these ratios are greater than the corresponding values for visible light; the ratio for visible light is



about 0.2 for a clear sky at sea level and  $Z = 30^\circ$ . The ratio, either for ultraviolet radiation or for visible light, increases with increasing haze, and in thick haze or cloud overcast, when the direct rays of the sun are reduced to zero, the ratio becomes infinite because there is still light from the sky.

The distribution of ultraviolet radiation for wave lengths less than 3200 Å over the sky was measured by Pettit (1932) during the spring of 1928. His average values of the ultraviolet sky brightness at Pasadena

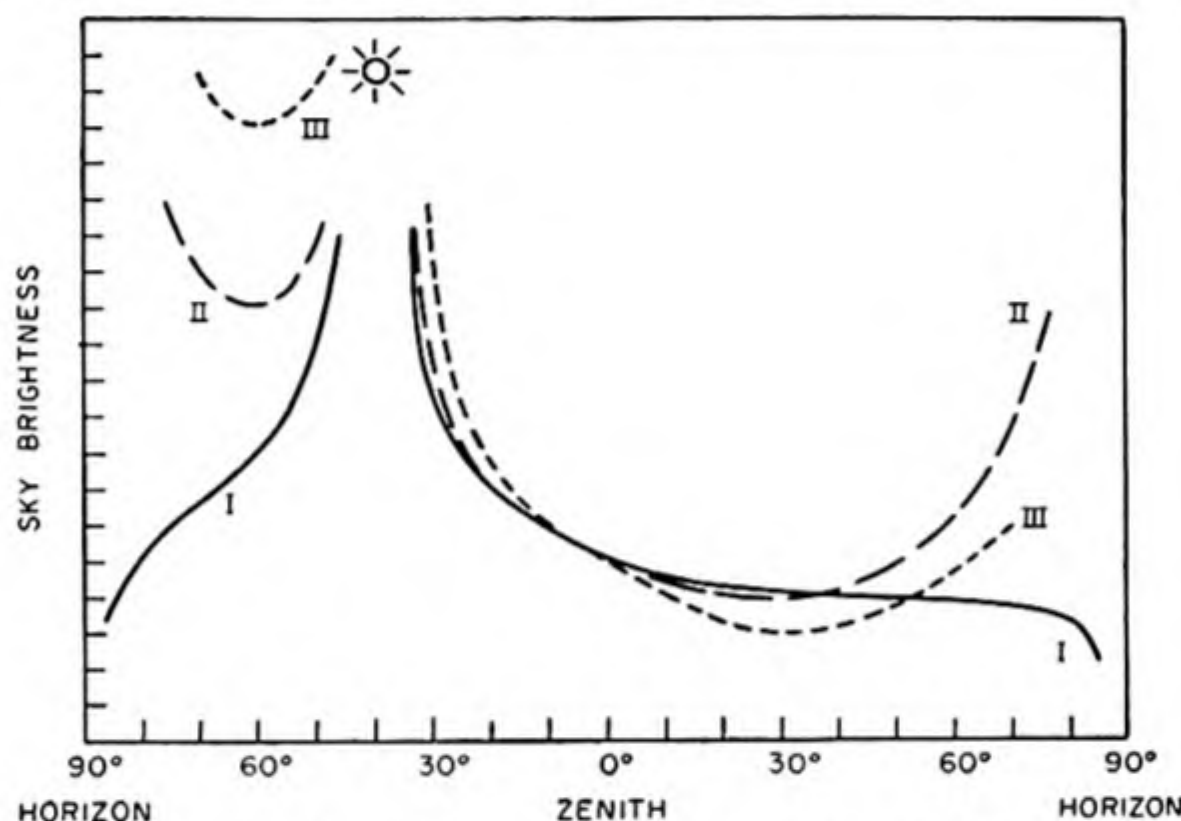


FIG. 3-13. Sky brightness for ultraviolet radiation and visible light. Curve I, for ultraviolet wave lengths less than 3200 Å in California. Curves II and III, for visible light in Brazil and Switzerland.

on a meridian through the sun with the sun at an altitude of  $50^\circ$  are shown by the solid-line curves of Fig. 3-13. The values varied considerably from day to day because of changes in haze. For comparison two curves of sky brightness for visible light are plotted in Fig. 3-13, one observed in Brazil (Richardson and Hulburt, 1949) and one in Switzerland (Dorno, 1919, Table IIId). The curves refer to a clear sky, to a meridian through the sun, and to the sun at an altitude of  $50^\circ$ ; the three curves are adjusted to pass through the same point at the zenith. The difference in the two visible-light curves was probably due to the haze conditions in the respective atmospheres. The curves show the well-known brightness of the sky near the sun and indicate that the sky near the horizon was relatively darker for ultraviolet radiation than for visible light.

### SOLAR INFRARED SPECTRUM AT THE EARTH'S SURFACE

In Fig. 3-14 is given the solar spectral-intensity curve  $i_0$  on top of the atmosphere and the transmission curves of the important terrestrial absorber, water, in liquid and vapor form as observed with spectrometers of low resolving power. The transmission curves for liquid water refer

to thicknesses of 1 and 10 mm of distilled water at 20°C calculated from the accurate absorption coefficients of Curcio and Petty (1951). The water-vapor curve represents the transmission through 1.85 km of atmosphere along a horizontal path containing some haze and a total of 17 mm of precipitable water. That is, the water vapor in a column 1 cm square and 1.85 km long would, if condensed to the liquid phase, form a column of liquid water 1 cm square and 17 mm long. The portion of the water-vapor curve for wave lengths longer than 0.9  $\mu$  was measured in

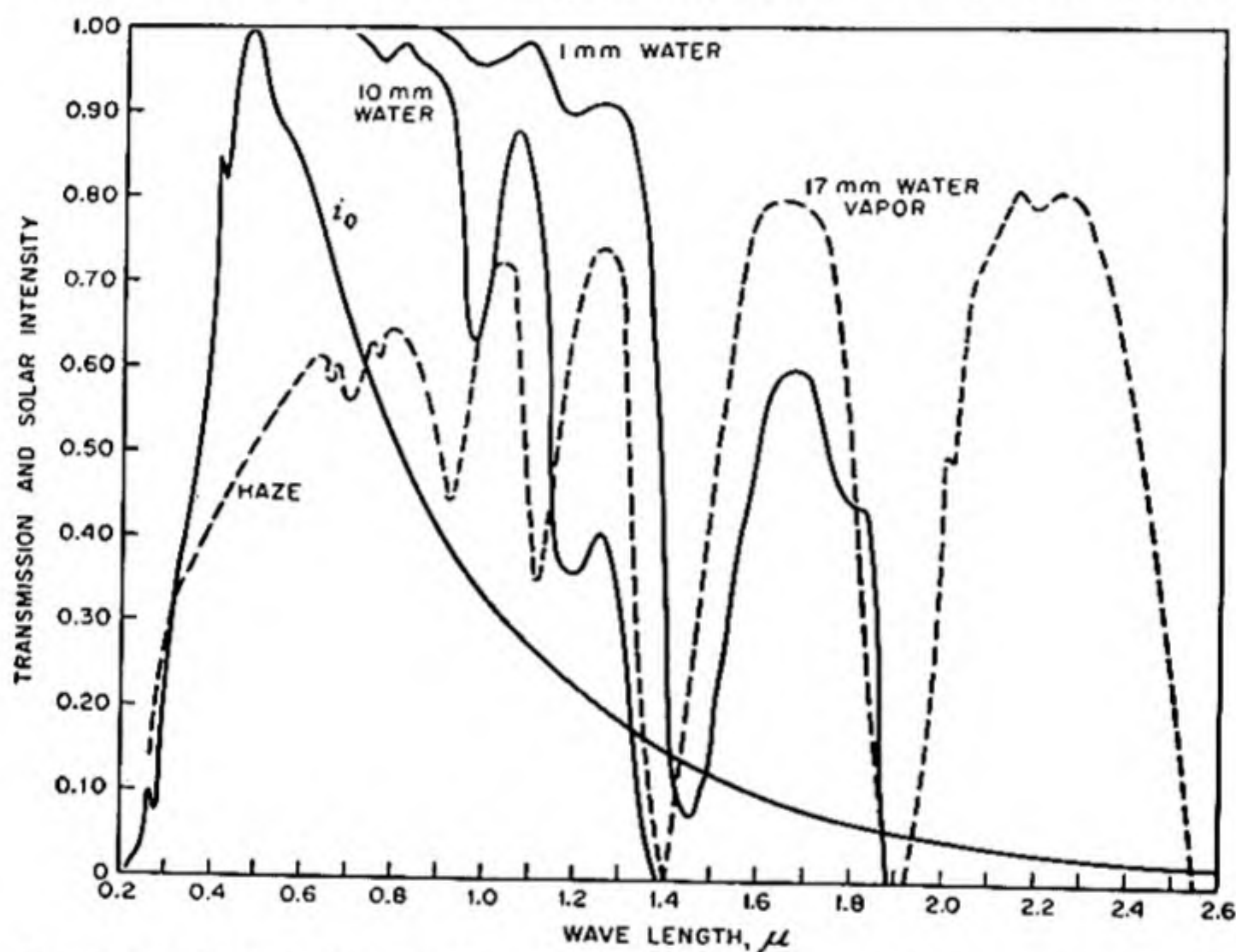


FIG. 3-14. Solar intensity  $i_0$  and transmission of water and water vapor.

1949 by Gebbie *et al.* (1950). The short-wave-length portion of the curve below 0.9  $\mu$  was from miscellaneous older measurements along horizontal paths in the real atmosphere; the attenuation below 0.6  $\mu$  was largely due to haze because water vapor is very transparent in this region.

The curves of Fig. 3-14 bring out the well-known differences in the absorption of water in the liquid and vapor phases. For example, beyond 1.4  $\mu$  the strength of absorption by liquid water is much stronger than by water vapor. Thus, 10 mm of liquid water and 17 mm of water vapor are opaque at 1.4  $\mu$ , but, although the transmission of water vapor rises at 1.65  $\mu$  to a high value, liquid water remains completely opaque at longer wave lengths. Also, it is seen that the absorption coefficients of 17 mm of water vapor and of 1 mm of liquid water are comparable on the short-wave-length side of the 1.9- $\mu$  water-vapor band, but, although water vapor regains its transparency at 2  $\mu$ , 1 mm of liquid water does not again become transparent. Furthermore, the wave lengths of maxi-



imum absorption do not coincide for the two phases. Approximately the same number of absorption bands exist at wave lengths below  $1.9\ \mu$ , but the vapor bands appear at somewhat shorter wave lengths than the liquid bands.

Therefore sunlight that reaches the earth through several centimeters of precipitable water vapor is not entirely depleted of energy capable of being absorbed by liquid water in organisms or in the seas, and, in particular, this is true in the so-called "atmospheric windows" at  $1.65$  and  $2.2\ \mu$ ,

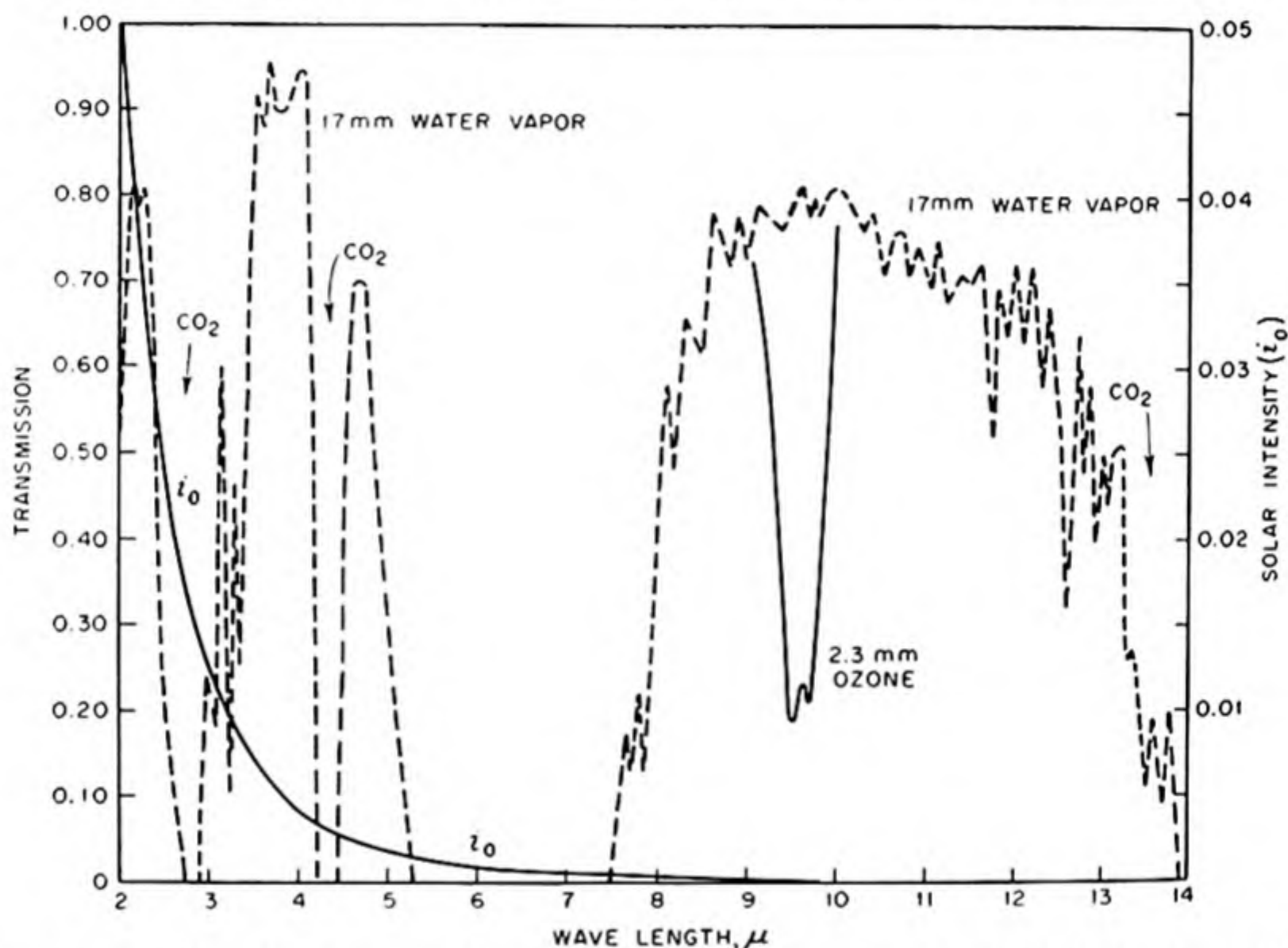


FIG. 3-15. Solar intensity  $i_0$  in the infrared and transmission of water vapor, ozone, and carbon dioxide.

where atmospheric water vapor transmits copiously and relatively small thicknesses of liquid water absorb strongly. The effect of absorption in the infrared is to heat the absorber. In the  $2.2\text{-}\mu$  region and at longer wave lengths where liquid water is highly absorbing, heating is produced principally at the surface. On the other hand, for shorter wave lengths below  $1\ \mu$ , the radiation penetrates to a greater depth before being completely absorbed and produces warming in depth.

Figure 3-15 is an extension of the solar-intensity and the atmospheric-transmission curves of Fig. 3-14 to  $14\ \mu$  to show other atmospheric infrared absorption bands. The absorption at  $2.7\ \mu$  is due to both carbon dioxide and water vapor. The strong absorption at  $4.2\ \mu$  is due to carbon dioxide, and the great band from  $5.2$  to  $7.5\ \mu$  is due to water vapor. Beyond this band to about  $14\ \mu$  the lower atmosphere is relatively trans-

parent except for the complicated but slight absorption of water vapor. The  $9.6\text{-}\mu$  infrared band of ozone of the upper atmosphere is shown. At  $14\text{ }\mu$ , a strong band of carbon dioxide sets in, and thereafter, for longer wave lengths up to about  $400\text{ }\mu$ , or  $0.4\text{ mm}$ , water vapor is a strong absorber with the exception of a narrow crevice of transmission at  $22\text{ }\mu$ . In conclusion, it is apparent that the curves of Figs. 3-14 and 15 can be used to make rough estimates, perhaps correct within a factor of 3, of the solar energy in the infrared at the surface of the earth if the amount of water vapor in the overhead atmosphere is known, but, if greater precision is required, provision must be made to measure the radiation directly.

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## CHAPTER 4

# Technique of Study of Biological Effects of Ultraviolet Radiation

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*Introduction. Sources: Classification of light sources—Physical parameters of sources—Choice of a source—Practical aspects. Detectors of ultraviolet radiations: Fluorescent screens—Thermal detectors—Photochemical detectors—Photographic detectors—Photoelectric detectors. Methods of spectral isolation: Filters—Dispersing systems. References.*

## INTRODUCTION

The technique of the study of the effects of any radiation on living systems is divisible, on an operational basis, into (1) the means of producing the radiation, (2) the means of manipulating and estimating the various parameters of the radiation, and (3) the means of demonstrating and analyzing the effects of the radiation on the biological system under study. This operational outline will be adhered to in a discussion of sources of ultraviolet radiation, detectors of ultraviolet radiation, means of spectral isolation, and accessory optical components. The various means of demonstrating and analyzing the effect of ultraviolet radiation on the biological systems are considered in great detail elsewhere in this volume and will not be taken up here. It must be emphasized that this chapter will not deal with the detailed technique of any particular study but will be concerned with materials for such an investigation. This approach is dictated by the great variety of problems in this field. For example, one investigator may be interested in the abiotic activity of the

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sunlight as a function of season, altitude, or some other parameter. A second investigator, using the same biological test object, might wish to determine the action spectrum for the lethal effect of ultraviolet radiation in great detail over a wide range of wave lengths. Each of these workers would draw from the same reservoir of available tools but would combine them in a different fashion for his own particular problem. The function of this chapter is to serve as a guide.

## SOURCES

The source of radiation is of great importance among materials for a study of effects of ultraviolet radiation on biological systems. A number of excellent chapters have been written containing detailed considerations of light sources in general and of the ultraviolet light sources in particular (see, e.g., Ellis *et al.*, 1941; Forsythe, 1937; Harrison *et al.*, 1948; Sawyer and Vincent, 1939; and Koller, 1952). No effort will be made to reproduce these detailed discussions in this chapter but rather to discuss types of light sources and their characteristics, factors to be considered in the choice of a light source, and finally to present a tabular compilation of noncommercial and commercial laboratory light sources which have characteristics making them particularly useful in a study of radiation effects.

## CLASSIFICATION OF LIGHT SOURCES

Sources of radiant energy have been classified in a number of ways among which are: (1) the spectral range of radiation of useful intensity, (2) the method used for exciting the radiation, and (3) the distribution of energy within the spectral range. The ultraviolet spectrum, which is discussed in this volume, covers the range 4000–10 Å. This broad range has been subdivided primarily on technical grounds into the near, the far, and the extreme ultraviolet. Sources differ considerably in the fraction of the total energy emitted in each of these ranges. The near ultraviolet extends to 3000 Å which is near the short wave-length limit for the sunlight at the earth's surface. The far ultraviolet in biological work extends to about 1900 Å. In this vicinity quartz begins to absorb strongly as does atmospheric oxygen (Schneider, 1940; Ladenburg *et al.*, 1932). Because of this increasing atmospheric absorption below 1900 Å, the extreme ultraviolet is also known as the vacuum ultraviolet. The lower limit of the extreme ultraviolet is arbitrary. There is considerable overlap in this region between the longer wave lengths of radiation produced by the techniques employed in the excitation of X radiation and those found by excitation of ultraviolet radiation.

*Method of Exciting the Radiation.* Ultraviolet radiation of any portion of the ultraviolet spectrum may be produced by any one or more of the following means of excitation: (1) incandescent or thermal, (2) spark,

(3) arc, or (4) discharge. The modern tungsten lamp is an excellent example of a source of the first category. The radiation takes place as a result of heating of the surface of the radiator by some means; in this case it is by the passage of an electric current. For fundamental reasons sources of this category have limited utility, and that only in the near ultraviolet. In most cases the intensity of the emitted radiation falls rapidly between 4000 and 3000 Å, approaching zero at the latter figure.

Spark sources emit radiation excited by the passage of a high-voltage discharge between electrodes. The material of the electrodes enters the spark stream, contributing the major fraction of the radiation through its excitation by the electrical energy. In the arc also the electrode material evaporates into the arc stream to produce a large portion of the emitting ions in this stream. Arcs are generally low-voltage, high current discharges. Radiation is produced in the discharge tube by excitation and ionization of the gas contained at reduced pressure. The radiation is excited by a relatively high potential between electrodes which, themselves, do not contribute significantly to the ion stream. The distinction between these methods of excitation is not sharp and the reader will find that the foregoing system of classification is not rigidly adhered to in the literature. This is understandable when it is noted that discharge tubes operated at very high current densities may show evidence of evaporation of the electrode material into the ion stream by the appearance of radiation characteristic of the electrode material. The heating of the electrodes of an arc by ion bombardment may be sufficient to make the thermal radiation from the electrode a significant contribution to the total radiation from the source. A spark operated in air produces radiation which is characteristic of the electrode material, but if operated under water, the radiation produced bears no relation to the electrode material.

*Spectral Distribution.* Of somewhat more practical importance is the classification of light sources with respect to the distribution of the spectral energy emitted. Sources are classified as continuous, line, or band. Continuous spectra generally arise from thermal emitters or from non-quantized energy transitions; line spectra arise from quantized atomic-energy transitions; and band spectra arise from molecular-energy transitions or from atomic-energy transitions occurring at high temperatures and pressures. On the basis of the method of production of these various types of spectra it would be expected that many sources would exhibit other than the nominal type of spectrum. Thus, when the hydrogen discharge tube is operated at extremely high current densities in an effort to achieve high brilliance, line spectra are frequently found superimposed upon the typical continuous ultraviolet spectrum of hydrogen. These lines arise from the evaporation and subsequent excitation of electrode material in the ion stream. Mercury discharge tubes, which at low pressures and current densities show well-defined line spectra, show increasing



broadening of the lines and the development of appreciable continuous background as the current density and vapor pressure are increased.

#### PHYSICAL PARAMETERS OF SOURCES

Two of the physical parameters which may influence the choice of a particular light source were mentioned in the preceding discussion of possible classification of sources, namely, the useful spectral range covered by the emitted radiation and the distribution of energy within that range (i.e., continuous or discontinuous). A third factor of importance is the amount of radiation emitted at a particular wave length or over a certain band of wave lengths. The amount of radiation may be considered in two ways: first, the total amount of radiation emitted from the whole of the luminous body of the source. Second, the amount of radiation may be considered to be that quantity emitted by a unit area or volume of the source into a unit solid angle. The significance of these two modes of expressing the intensity parameter of a light source will be discussed. Following is a list of the terms which are used to describe the intensity parameter:

1. Radiant Flux: Radiant flux is the rate of flow of radiant energy with respect to time. The quantity is also called "radiance" ( $P = dU/dt$ ) where  $U$  is radiant energy and  $t$  is time.

2. Radiant Intensity: Radiant intensity is measured by the energy falling in unit time upon an area subtended by unit solid angle about any direction considered and at any distance from the source. This value is also called "steradiancy" ( $J = \frac{dU}{dt d\omega}$ ). The solid angle is represented by  $\omega$ .

3. Steradiancy: Steradiancy is the radiant flux per unit solid angle per square centimeter of source ( $B = \frac{dU}{dt dA d\omega}$ ).  $A$  is the area of the source.

It is to be noted that these expressions contain no reference to wave length. For studies of the biological effects of radiation it is frequently necessary to know the value of one or more of the preceding intensity expressions with respect to wave length. The manner in which such energy measurements are made will be considered on pages 130 to 142.

#### CHOICE OF A SOURCE

The choice of a source for a study of the biological effects of radiation depends on a number of factors which are inherent in the exact nature of the experiment to be conducted. In a general way, decisions must be made as to (1) the size of the area to be irradiated, (2) the range of the ultraviolet spectrum to be covered, (3) the size of the band of energy with respect to wave length, (4) the time in which the largest amount of energy is to be delivered to the irradiated area. These factors are to some extent

independent and yet they are often interdependent in a way which is not always completely understood. This situation can probably be best presented by examples. Assume the practical problem of the sterilization of large volumes of a liquid by ultraviolet radiation. This is to be done by flowing the liquid in a thin film of large area exposed to the total radiation exclusive of the infrared. For practical reasons it is decided to use the mercury-vapor discharge lamp. The amount of ultraviolet energy needed has been established by previous experiments.

The mercury discharge lamp has a relatively large fraction of the total ultraviolet output in the biologically potent region around 2600 Å but the intrinsic brilliance (steradiancy) is quite low compared with other lamps. This particular problem allows a large area into which the required energy can be delivered. Thus what the mercury discharge tube lacks in steradiancy can be made up by extending the emitting area, which is quite easily accomplished, until the total amount of energy received by a unit volume of liquid during exposure meets the experimental requirement.

The broad band of radiation to be used in this experiment is most easily isolated by a filter system which can usually also be extended in area at will (see p. 142).

It is quite clear that, in this case, it would have been uneconomical and difficult to have attempted the use of a source which was very bright, i.e., of high steradiancy. Such sources usually attain brilliance by high current densities in small volumes. The total energy output may therefore be less than a greatly extended source of low steradiancy. Indeed one of the highest rates of total ultraviolet output has been achieved with such a source of low steradiancy. Furthermore, as can be seen in the general references cited, a high current density is often obtained at the expense of simplicity and ease of operation.

The steradiancy of a source becomes a matter of importance when for any reason it becomes necessary to use an image of the source for irradiation. The whole matter of power transmission through image-forming systems has been considered in detail by Loofbourow (1950) and Blout *et al.* (1950).

The importance of a careful study of these principles may be indicated by the example which follows. Assume that only the cytoplasm of a cell is to be irradiated and a study made of the effects of such irradiation on the nucleus. Such an experiment will require the formation of a reduced image of the source or a portion thereof within the cytoplasm. This would probably be accomplished by use of a reflecting objective as a condenser. In these experiments also the use of the total emission of a source will be assumed. The following relation, known as Lagrange's Law (see Hardy and Perrin, 1932, p. 43), has been shown to hold by a number of writers including those cited.



The ratio of area in object (source) space  $A_1$  to that in image space  $A_2$  is equal to the ratio of the solid angle of rays forming the image and leaving the source, or expressed in terms of linear dimensions,

$$L_1NA_1 = L_2NA_2,$$

which says that the product of a linear dimension of the source and the numerical aperture of the rays leaving the source collected by the imaging system is equal to the product of the same dimension of the image of the source and the numerical aperture of the image-forming rays.

Let us say that we wish to form an image of the source which is 0.005 mm in diameter with a condenser lens of  $NA = 0.5$ . Thus,

$$0.005 \times 0.5 = 0.0025.$$

We assume a field lens for the source which has as high an aperture as the condenser. Then,  $0.0025 = L_10.5$ .

$$L_1 = 0.005 \text{ mm.}$$

From this it is clear that only an area of the source of diameter 0.005 mm is contributing to the energy flowing into the cytoplasm. Should a collecting lens of smaller numerical aperture be used, a larger area of the source would contribute, but through a smaller solid angle and, assuming the source to be uniform, the total energy would be the same. In order to increase the amount of energy delivered into the cytoplasm in a given time the product  $L_2NA_2$  must be increased, the steradiancy of the source must be increased, or both.

This fundamental relation has been demonstrated in an example of simple image formation. It has been shown to hold as the limit no matter how many image-forming steps are interposed between the source and the final image used for irradiation.

Consider, for example, a possible arrangement of optical components (Fig. 4-1) for the determination of the action spectrum (see p. 384) of the effects of ultraviolet radiation found in the preceding experiment. In this system  $S$  is the source focused by a collecting lens on  $SL_1$ , the entrance slit of the monochromator  $M$ .  $SL_2$  is the exit slit of the monochromator and  $I$  the image formed in the cytoplasm by the condenser. Then,

$$L_SNA_S = L_{SL_1}NA_M = L_{SL_2}NA_M = L_INA_I.$$

In this case the slits of the monochromator serve as secondary sources. The width of the slits ( $L_{SL_1}$ ,  $L_{SL_2}$ ) is determined by the dispersion of the monochromator and the required width of the band of radiation to be isolated (p. 148). This may establish a limiting value for the products including these terms because of limitations inherent in available monochromators. It is again emphasized that the establishment of a value for any one of the products  $L_xNA_x$  by experimental requirements or by

instrumental limitations establishes the value for the remaining products and thereby the technical requirements for all other optical components in the system.

This treatment has been limited but is intended to indicate the framework within which an intelligent choice of a source of radiant energy is made.

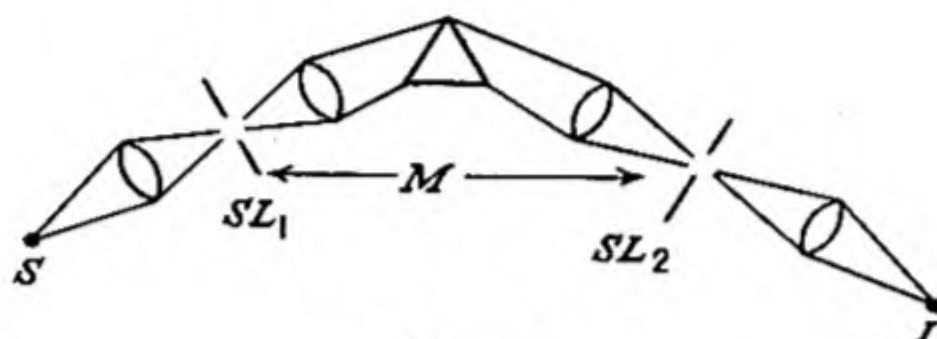


FIG. 4-1

#### PRACTICAL ASPECTS

The last important group of factors in the choice of a particular source are the practical considerations which include simplicity of construction and operation, ruggedness, useful life, availability, and cost. In the past two decades the commercial availability of many ultraviolet sources together with the power supplies for their operation from domestic mains has increased greatly. Because of the importance of the ready availability of many sources a separate section has been given over to the description of these (see p. 126).

To avoid repetition of the descriptive data contained in the general references noted earlier in this section, Table 4-1 has been prepared. This table constitutes a summary of the various types of experimental and laboratory commercial light sources which have been found useful in radiation studies in the ultraviolet range of the spectrum. No effort has been made to summarize all the data on the subject, but rather to give leads to the literature on some of the older sources which have certain useful characteristics and to give more detailed data on the more recent developments. The reader is again advised to consult the following references for a detailed description of the older sources of ultraviolet light: Forsythe, 1937; Ellis *et al.*, 1941; Harrison *et al.*, 1948; and Koller, 1952.

#### DETECTORS OF ULTRAVIOLET RADIATIONS

Ultraviolet radiations may be observed by the use of fluorescent screens, or such radiations may be detected and quantitatively measured by means of the thermal, chemical, or electrical effects they produce. Visual detection is of value in instances where qualitative observations are adequate, as in the alignment of optical systems; thermal and photochemical effects may be employed to determine absolute quantities of radiation; because of their susceptibility to amplification, some photochemical



TABLE 4-1. ULTRAVIOLET LIGHT SOURCES

Mode of production of radiation	Spectral range (UV)*	Spectral distribution	Energy output†	Source size	Power requirements	Life of the source	Special comments	References
Disruptive spark (exploded wires)	Whole	Continuous with self-absorption bands 15,000°K	Intrinsic brilliance 100 times that of the sun. Flash lasts 10 <sup>-4</sup> sec	5 cm long × 2.2 cm diameter	26,000–50,000 volts 0.3–0.4 capacitance	One explosion	Very noisy and moderately destructive. Heat effects absent	Anderson, 1920; Forsythe and Easley, 1931; Sawyer and Becker, 1923
Underwater spark	Near and far	Continuous 10,000°K	Very high intrinsic brilliance	14–16 mm long × 4 mm diameter	20,000 volts 1 kva	(See references)	Spectrum independent of electrode material. Continuous supply of water necessary	McNicholas, 1928; Wyneken, 1928; Wrede, 1929
Thermal sources	Near	Continuous, ca. 3000°K	(See references)	(See references)	(See references)	(See references)	Useful only in near ultraviolet	Holladay, 1928; Brockman, 1947
Vacuum spark	Whole	Continuous	(See references)	(See references)	30,000–150,000 volts/mm of gap at 10 <sup>-4</sup> to 10 <sup>-8</sup> mm of Hg pressure	(See references)	Primarily a source for the extreme ultraviolet	Millikan and Sawyer, 1918; Millikan, 1920; Sawyer, 1920; Carter, 1922; Edlén, 1936; Anderson, 1924
Metallic spark	Near and far	Discontinuous	(See references)	(See references)	(See references)	(See references)	Somewhat noisy and inconvenient, but sometimes used as a source of intense monochromatic radiation when a large fraction of radiated energy is in a few well-separated lines	Forsythe, 1937

Open arc (carbon)	Near	Continuous with superimposed emission lines which depend on the core material	Depends on current, not on the carbon size (see reference)	2-5 mm diameter (see reference)	(See references)	(See references)	High intrinsic brilliance	Nottingham, 1926; Forsythe, 1937; Harrison <i>et al.</i> , 1948; MacPherson, 1940; King, 1925; Koller, 1952
Enclosed arcs (metallic)—Hg, Bi, Cd, Pb, Zn, Ti, Zr	Near and far	Discontinuous. Lines depend on metal used. Line width and level of background continuum depend on vapor pressure	Extensive detailed data on spectral distribution of radiant energy given in references	20-50 mm long $\times$ 1-3 mm diameter	ca. 100 volts (running voltage) 8-10 A maximum current	Hg: 25-30 hr Other metals: 10 hr	Easily constructed. Water cooled. High intrinsic brilliance. Lamps containing other metals than Hg and Zr can be used but once. Zr arc is a commercial lamp	Crist, 1931; Daniels and Heidt, 1932; Hoffman and Daniels, 1932; Anderson, 1943; Coolidge, 1944; Elenbaas, 1948; Koller, 1952
Enclosed arc (gas)—Xe	Near and far	Continuous, 5200°K	Extensive, detailed data on spectral distribution of radiant energy given in references	80% of the radiation from area of 10 mm. <sup>2</sup> 50% of radiation from area of 4.2 mm <sup>2</sup>	125- and 500-watt sizes available	(See references)	Commercially available sources. (See footnotes a)	Schulz, 1947a, b, c; Aldington, 1949; Baum and Dunkelman, 1950
Enclosed arc (gas)—H	Near and far	Continuous up to ca. 3700 A	See Matz and Merrill (1949) for data on sources <sup>a,b,c</sup> . Source <sup>d</sup> is intermediate between <sup>a</sup> and xenon arc (9) in intrinsic brilliance	From a few mm to 20 cm in length 1-5 mm diameter	(See references)	More than 100 hr	Commercially available sources (See footnotes a, b, c, d, e)	Kistiakosky, 1931; <sup>a</sup> Allen, 1941; <sup>b</sup> Allen and Franklin, <sup>c</sup> 1939; Finkelstein, 1950; <sup>d</sup> Matz and Merrill, 1949 <sup>a,b,c</sup>



TABLE 4-1. ULTRAVIOLET LIGHT SOURCES.—(Continued)

Mode of production of radiation	Spectral range (UV)*	Spectral distribution	Energy output†	Source size	Power requirements	Life of the source	Special comments	References
Discharge tube Xe, Hg— continuous operation	Whole	Discontinuous. Lines depend on gas used. Line width and level of background continuum de- pend on pressure	See Buttolph (Chap. 2, this volume) for data on Hg sources $2 \times 10^{13}$ to $10^{16}$ quanta/sec at 1470 Å (Xe) $6 \times 10^{15}$ quanta/sec at 1850 Å (Hg)	(See references)	5000–6000 volts 0.1–0.2 A	ca. 100 hr	Used with white sapphire win- dows for trans- mission of 1470 Å line of Xe	Calvert, 1932; Groth, 1937; Freed <i>et al.</i> , 1939; Ramasastry, 1947; Dacey and Hodgins, 1950
Discharge tube Xe, Hg— pulsed operation	Near and far	Continuous	$10^{10}$ to $10^{14}$ quanta/sec in uranyl oxalate absorption band. Total output depends on length of the source	Lengths up to 1 m have been used. Discharge col- umn is ca. 4 mm in diameter	1000–35,000 volts 2 $\mu$ F capaci- tance Current density 30,000–100,000 A/cm <sup>2</sup>	(See references)	Flash duration $10^{-4}$ to $10^{-8}$ sec	Anderson, 1932; Edgerton and Ger- meshausen, 1932; Edgerton <i>et al.</i> , 1937; Edgerton, 1946; Norrish and Porter, 1949

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\* Adam Hilger, Ltd., London, England.

\* The useful spectral range of the ultraviolet is noted. The wave-length limits of the near, far, and extreme ultraviolet are given on page 120.

† Where detailed data on energy output are available, this is noted. Where feasible, any indication of the energy output of a source given in the literature has been noted.

(photographic) effects and the electrical effects make possible the measurement of small quantities of such radiations.

#### FLUORESCENT SCREENS

A great variety of substances fluoresce under ultraviolet irradiation (DeMent, 1945). The properties of certain of these, of especial interest to the lamp industry, have been studied in considerable detail (Kröger, 1948; Fonda and Seitz, 1948).

Zinc silicate (willemite) powder can be used to make very satisfactory fluorescent screens over the wave-length region 1000–3000 Å (Beese, 1939; Lui, 1945). Maximum excitation is obtained with radiation near 2500 Å, for which the quantum efficiency of fluorescence is nearly unity (Fonda, 1939; Schulman, 1946). The emission spectrum of manganese-activated zinc silicate peaks at 5250 Å but the emission can be shifted throughout the visible spectrum by addition of beryllium and of various activators (Leverenz and Seitz, 1939). Throughout the region 2200–3000 Å nonabsorbent silicone resins, such as General Electric #9980, may be used as a binder. Magnesium tungstate may also be used as a phosphor throughout the wave-length region 2200–3000 Å with a quantum efficiency nearly unity (Fonda, 1944; Oszy, 1951). Data on other phosphors useful on this spectral region are summarized by Thayer and Barnes (1939).

For the 3000–4000 Å region, sulfide phosphors are quite effective (Klasens *et al.*, 1948; Studer and Larson, 1948; Pringsheim, 1949, pp. 582ff., pp. 594ff.) with high quantum efficiency about 3650 Å (Leverenz and Seitz, 1939). A large number of varicolored pigments are known which respond to radiation in this region (Barnett and Grady, 1949).

Special phosphors have been developed to convert ultraviolet radiation at 2537 Å to ultraviolet radiation at other wave lengths for particular purposes (Froelich, 1947). Thus ultraviolet-sensitive phosphors emitting radiation in the erythematous region (2900–3200 Å) (Clapp and Ginther, 1947; Nagy *et al.*, 1950) and the "black-light" region (ca. 3600 Å) (Beegs, 1943; Clapp and Ginther, 1947) have been described.

Fluorescent coatings may be employed to extend the usefulness of phototubes to wave-length regions shorter than the transmission limits of their envelopes. Déjardin and Schwégler (1934) used sodium salicylate to extend the effective range of a potassium hydride surface phototube from 3400 to 2200 Å. A constant quantum efficiency of fluorescence was obtained over this region. Coatings of salicylate and other materials have been used to extend the sensitivity of photomultiplier tubes to 900 Å in the vacuum ultraviolet (Johnson *et al.*, 1951). Again a constant quantum efficiency was found with salicylate, independent of the wave length of excitation.

It would seem quite feasible to make use of the threshold wave lengths,



and spectral variation of sensitivity of various phosphors, in conjunction with the spectral response of phototubes, to make wave-length-selective detectors (e.g., Luckiesh and Taylor, 1940; Kerr, 1947).

#### THERMAL DETECTORS

The measurement of radiant energy in absolute units is most commonly accomplished by the total absorption of such energy in an appropriate substance, accompanied by a measurement of the increase in temperature of the absorber. If the heat capacity of the absorber is known, the energy content of the radiation may then be readily calculated. In practice, the increase in temperature of the absorber may be measured as a resultant change in electrical resistance (bolometer), as an electromotive force (thermocouple), or as a mechanical deformation induced by gas expansion (Golay cell). Radiation detectors based on a measurement of the change in temperature consequent to radiation absorption are called thermal detectors.

To absorb totally the radiant energy, the detector must be "black" to all wave lengths represented in the radiation to be measured. It is possible to prepare such "black" surfaces by vacuum deposition, under appropriate circumstances, of such substances as bismuth, zinc, platinum, or gold (Pfund, 1930, 1933, 1937a,b; Harris and McGinnies, 1948). Such surfaces are known to absorb all incident radiation from  $0.2\ \mu$  to beyond  $15\ \mu$ .

These detectors may be calibrated by the use of a radiation beam of known energy content, thereby avoiding the necessity of a direct measurement of their heat capacity. Such a defined beam may be obtained from standard lamps, available from the U.S. Bureau of Standards (Coblentz and Stair, 1933), operated under precisely defined conditions.

*Bolometer.* Measurements of the change in electrical resistance of a detector, consequent to the absorption of radiant energy, are conveniently carried out by using the detecting bolometer as one arm of an initially balanced resistance bridge. The change in resistance leads to unbalance of the bridge with a resultant unbalance voltage which may be amplified to a readily meterable magnitude. A similar bolometer, shielded from the radiation, may be placed in an appropriate arm of the bridge to compensate for variations in ambient temperature. If the thermal capacity of the detector is low, it may be used with chopped radiation, with a resultant oscillatory unbalance voltage, which may be amplified by an alternating-current amplifier; in such cases the amplifier may well be sharply tuned to the chopping frequency to improve the signal-noise ratio.

To produce a bolometer detector of high sensitivity, it is desirable to deposit the absorbing coating upon a substance with a high temperature coefficient of resistance. Among the metallic substances, nickel and

platinum (0.3 and 0.6 per cent per degree Centigrade, respectively) have been used. The semiconductor thermistors have appreciably larger, negative coefficients of resistance (to  $-5$  per cent per degree Centigrade) (Becker *et al.*, 1946) and are widely used (Dodd, 1951). Both thermistor and metallic bolometers have been made with a limiting sensitivity of about  $10^{-8}$  watt, with a response time of a few milliseconds (Baker and Robb, 1943; Jones, 1946; Billings, Barr, and Hyde, 1947; Billings, Hyde, and Barr, 1947; Schlesman and Brockman, 1945), or increasing sensitivity to  $10^{-10}$  watt with response time of a few seconds (Jones, 1949).

**Thermocouple.** By placing the absorbing surface in good thermal contact with a bimetallic junction, a change in the temperature of the absorber may be measured as a change in the potential difference across the junction. To minimize the effects of ambient temperature changes on such a thermocouple detector, this potential is usually measured with reference to the potential across a similar junction, in thermal contact with a second absorbing surface, adjacent to the first, but not exposed to the radiation beam. If several such pairs of junctions are connected in series to produce a larger total change in potential difference, the device is called a thermopile.

The potential difference thus developed may be amplified in a direct-current amplifier, or it may be mechanically interrupted in a "breaker" amplifier and thereby converted into an oscillatory signal to be amplified in an alternating-current amplifier (Liston *et al.*, 1946). Alternatively, if the absorber has a small heat capacity, the radiation beam may be mechanically chopped to provide a cyclic voltage. This latter method has the additional advantage that it minimizes the effects of slow "drifts" between the potentials of the measuring and reference junctions.

The rate of change of potential difference across such a junction with change of temperature is the thermoelectric power of the junction. The highest thermoelectric powers are obtained with junctions between bismuth-antimony and bismuth-tin alloys (Pfund, 1937a; Hornig and O'Keefe, 1947); with such junctions, powers of  $120 \mu\text{V}/^\circ\text{C}$  may be obtained. However, because of the fragility of bismuth alloy junctions, other metallic couples such as constantan-chromel ( $77 \mu\text{V}/^\circ\text{C}$ ) are occasionally used (Launer, 1940).

With such bimetallic couples and with careful design, it is possible to measure a rise in temperature of the absorber of the order of  $10^{-6}^\circ\text{C}$ . Such radiation detectors can provide a sensitivity of  $50 \mu\text{V}/\mu\text{W}$  or greater (Schwarz, 1949; Jones, 1949).

For use in photochemical experiments a thermopile with a large receiving area may be desired (Crane and Blacet, 1950).

**Golay Detector.** The Golay radiation detector is characterized by both a high sensitivity and a relatively rapid response time (Zahl and Golay, 1946; Golay, 1947a, b). The radiation is absorbed in a blackened surface



of low heat capacity immersed in an atmosphere of xenon in a small chamber. The rear wall of the chamber is a thin collodion film, silvered on the outside surface. When exposed to radiation, the absorbed heat is rapidly transferred to the gas, which expands, deforming the collodion wall. This slight deformation is readily detected by an optical system whereby an image of a grid is caused to move across another grid, varying the light received on the face of a photocell, as the membrane is deformed. The detector is intended to be used with chopped radiation. With such a cell, energies of  $5 \times 10^{-11}$  watt may be detected (Golay, 1949).

*Ultimate Sensitivity of Thermal Detectors.* The sensitivity of any thermal detector of radiation is ultimately limited by the random fluctuations to be expected on thermodynamic grounds, in the temperature of any body in equilibrium with its environment (Myers, 1946; Jones, 1947). It would be impractical to attempt to measure a temperature change due to incident radiation, which is small compared to these random fluctuations. Alternatively, from a different but equivalent point of view, one may regard the sensitivity as limited by the inevitable statistical fluctuations in the heat radiation emitted and received at all times by any body. Since a thermal detector is sensitive to radiation of all wave lengths, it is sensitive to the thermal radiation emitted by its surroundings. Any attempt to detect a radiation beam of energy less than the fluctuations to be expected in the thermal radiation energy received from (and emitted to) the surroundings (Fellgett, 1949) would be impractical.

Such considerations set a lower limit to the sensitivity of thermal detectors exposed to a surround at ordinary temperatures, at about  $3 \times 10^{-12}$  watt for a detector of area  $1 \text{ mm}^2$  and response time of 1 sec (Jones, 1947). To achieve higher sensitivity in any radiation detector, it is necessary to limit the wave-length region to which it is sensitive, in order thereby to reduce the fluctuation in the detector output, due to the fluctuation in incident thermal radiation energy. Thus, for instance, for objects at ordinary laboratory temperatures, the intensity of emitted thermal radiation of wave length less than 6000 Å is negligible. Therefore, a photoelectric detector which is sensitive only to radiations of wave length less than 6000 Å is entirely insensitive to the fluctuations in thermal radiation.

#### PHOTOCHEMICAL DETECTORS

If monochromatic radiation is employed or if the spectral energy distribution of the radiation concerned is known, photochemical processes may be conveniently used as a measure of radiation intensity. For absolute determinations, the quantum yield of the photochemical reaction must first have been determined at all wave lengths of interest by calibration against a standard thermal detector. The use of a photochemical

detector, instead of direct use of a thermal detector, is frequently found advantageous for reproducing the geometry of an irradiation experiment, or for reasons of economy and convenience.

In ultraviolet, the photochemical decomposition of uranyl oxalate is widely used as an actinometer (Leighton and Forbes, 1930; Forbes and Heidt, 1934; Bowen, 1946; Launer, 1949). The quantum yield for this decomposition has been carefully measured at a series of wave lengths from 208 to 434  $m\mu$  (Leighton and Forbes, 1930; Brackett and Forbes, 1933). The initial oxalate content of the stock solution, and the residual oxalate after irradiation, are measured by permanganate titration and the decomposition determined by difference. From the quantity of oxalate decomposed and the quantum yield, the number of photons absorbed can be calculated, and from this figure, the incident intensity determined, knowing the absorption of the solution and making appropriate corrections for such factors as reflections at cell windows. It is convenient to use a solution of sufficient concentration to absorb all the incident radiation; adequate stirring must be provided to equalize the irradiation of all volumes of the solution. The photochemical reaction has been shown to have nearly unity temperature coefficient.

A rather unusual photochemical transformation of certain triphenylmethane dyes (Lifschitz, 1919; Lifschitz and Joffé, 1921; Harris *et al.*, 1935) from a colorless to colored form has been used as a means of comparing ultraviolet intensities in the spectral region 2400–3200 Å (Calvert and Rechen, 1952; Harris and Kaminsky, 1935; Weyde, 1930; Weyde *et al.*, 1930; Miyake, 1949). The quantum yield for the transformation has been shown to be unity over this spectral region and is independent of temperature (Weyde and Frankenburger, 1931; Calvert and Rechen, 1952). This actinometer can be used at considerably lower light intensities than can the uranyl oxalate. Under appropriate conditions, the transformation is quantitatively autoreversible, permitting the same solution to be used repeatedly (Weyde, 1930; Weyde *et al.*, 1932).

#### PHOTOGRAPHIC DETECTORS

The photographic plate or film has a number of advantages as a radiation detector. It is a sensitive detector and can integrate the radiation received. Furthermore, it is unique<sup>1</sup> among ultraviolet radiation detectors in that it is a two-dimensional detector, thus permitting an entire spectrum or microscopic image to be recorded in one exposure. While it cannot be regarded as a precision instrument for the measurement of radiation, with appropriate auxiliary techniques, accuracies of the order of  $\pm 3$  per cent can be obtained.

Since individual plates or films vary unavoidably in sensitivity and in contrast, for quantitative work it is necessary to standardize each plate

<sup>1</sup> With the potential exception of the image orthicon tube.



(for a general description of the properties of photographic materials, see James and Higgins, 1948). Furthermore, since the sensitivity and contrast vary with wave length, the plate must be calibrated at all wave lengths for which measurement is desired. (For data on the ultraviolet characteristics of spectroscopic plates, see Harrison, 1925a; Jones and Sandvik, 1926; Johnson and Hancock, 1933; Amstein, 1944. Fraser, 1950, has obtained data on the ultraviolet characteristics of several types of motion picture film.)

For homochromatic photometry, in which the only desire is to compare radiation intensities at a given wave length, the plate or film may be calibrated by means of any device which produces a scale of plate blackening versus source intensity on which the desired unknown intensities may be



FIG. 4-2. Rotating sectors: (a) stepped, (b) continuously variable. (*Reproduction from Practical Spectroscopy*, by G. Harrison, R. Lord, and J. R. Loofbrouow, Prentice-Hall, Inc., 1948.)

read (Harrison, 1934b). Calibrated step wedges or rotating sectors are commonly used for this purpose. Thin films of platinum (Merton, 1924; O'Brien and Russel, 1934; Uber, 1939) or Chromel A evaporated onto quartz (Banning, 1947a) are frequently used for the former, since they are nearly constant in optical density over a wide range of wave lengths, but for accurate work they must be calibrated. The rotating sector may either be stepped or it may vary continuously in exposure time (Fig. 4-2). It is truly a "neutral-density" device.

For heterochromatic photometry, in which it is desired to compare radiation intensities at different wave lengths, the relative sensitivity of the plate as a function of wave length must be determined. This is most easily done with a source of previously determined spectral energy distribution, preferably one with a spectral continuum, such as the hydrogen discharge tube.

Ordinary photographic plates are sensitive to ultraviolet radiations to wave lengths as short as 2300 Å. The faster of these plates require a net exposure to 0.1–1.0 ergs/cm<sup>2</sup> to produce a plate density of 0.5–1.0. Below 2300 Å, the absorption of the gelatin matrix for the silver halide grains prevents the radiation from penetrating beyond the upper layer of

emulsion and thereby greatly reduces the plate sensitivity. For work at shorter wave lengths, very thin emulsions heavily laden with silver halide grains may be used, such as the Ilford "Q" plates or the Eastman Kodak SWR film (Schoen and Hodge, 1950). As an alternative, the surface of the film may be coated with material that will fluoresce under the short-wave-length radiation, so that the exposure is actually produced by the fluorescent radiation (Harrison, 1925b). Such thin-emulsion or fluorescent-coated plates may be used far into the vacuum ultraviolet (Harrison and Leighton, 1930). By the use of a fluorescent coating with constant quantum yield of fluorescence, independent of exciting wave length, problems of heterochromatic photometry may be greatly simplified. Such coatings also eliminate the variation of contrast with wave length (Harrison and Leighton, 1931). For photomicrography, however, the use of fluorescent coatings generally leads to some loss of plate resolution.

#### PHOTOELECTRIC DETECTORS

Photoelectric detectors useful in the ultraviolet are of two general types: the photovoltaic or barrier-layer cell, and the photoemissive detector. The photovoltaic cells, which do not require an external power source, are convenient and useful in instances where relatively large amounts of radiant energy are available. The photoemissive detectors require more elaborate accessory equipment but are far more sensitive and are effective in a wide variety of applications.

*Photovoltaic Detectors.* Upon illumination of a photovoltaic cell, a potential difference appears across a semiconductor (usually iron selenide), which potential can be used to drive a current through an external circuit (Lange, 1938; Zworykin and Ramberg, 1949, Chap. 11). Electrically, the photovoltaic cell acts as a source of current which is shunted by an internal resistance and capacitance. The shunting internal resistance is not constant, but decreases with increasing illumination and with increasing current flow. Although the photocurrent generated within the semiconductor is, at moderate light levels, linearly dependent on light intensity, because of the internal resistance and its variation with light intensity, the external current is a linear function of light intensity only if very low external resistance is employed (Wood, 1934). As a consequence, the output of a barrier-layer cell as a function of light intensity, with various external resistances, is as shown in Fig. 4-3.

Electronic circuits have been developed to permit the use of larger external resistance, if desired for purposes of amplification, without introducing appreciable nonlinearity. Such circuits (Rittner, 1947) employ negative feedback to effectively reduce the apparent resistance external to the photocell.

The internal capacitance of the photovoltaic cell also acts to shunt the external resistance if an oscillatory photocurrent is produced by a modu-



lated light beam. This capacitance shunt limits the useful range of modulation frequencies to below 10,000 cycles/sec.

Although all commercially available barrier-layer cells have peak sensitivity in the visible spectral region, they are available in quartz envelopes which permit appreciable response to wave lengths as short as 270 m $\mu$ . These cells are somewhat temperature sensitive, and may display an initial "fatigue" for 15–20 min on exposure to radiation (Lange, 1938;

Barbrow, 1940). For use in appropriate applications, matched pairs of cells are available.

*Photoemissive Detectors.* Because of their high sensitivity, linearity and speed of response, and convenience of operation, photoemissive detectors have become the most widely used means for quantitative measurement of ultraviolet radiation. The electric currents derived from these devices are easily amplified and may then be used to operate meters or any of various kinds of automatic recording devices. With modern techniques (Engstrom, 1947a; Sommer and Turk, 1950) it is possible to reduce the extraneous sources of electrical fluctuation, such as the thermal emission of electrons from the photocathode and the thermal motion of electrons in the amplifier input circuit, to levels sufficiently low for the principal limitation on the precision

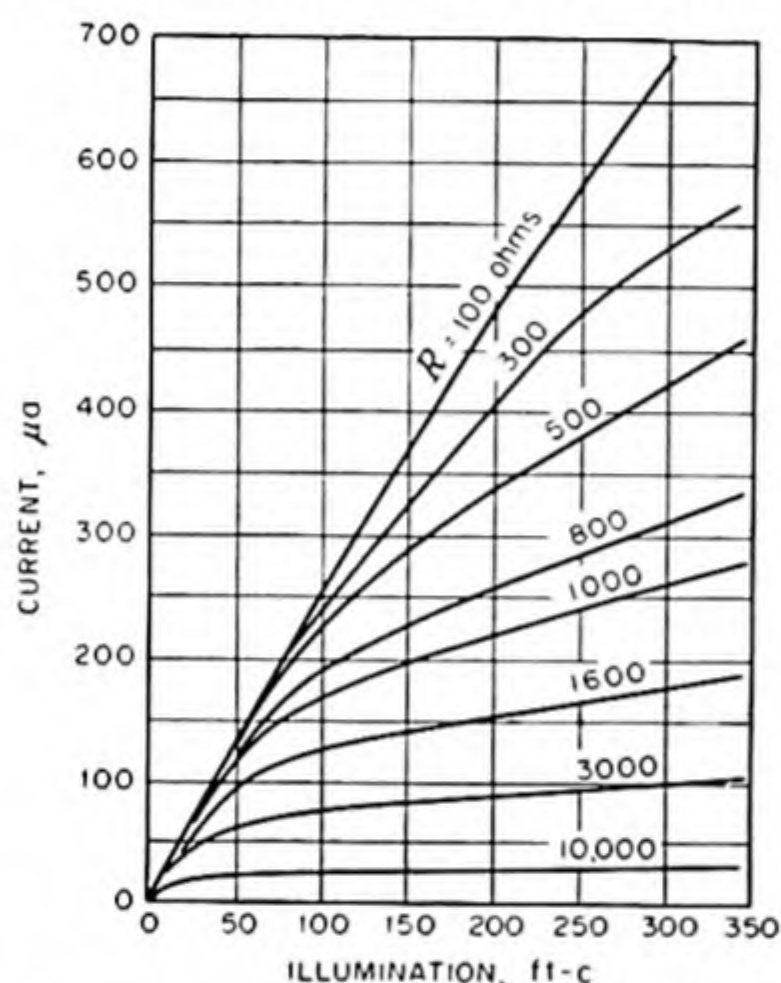


FIG. 4-3. Influence of external circuit resistance upon current output of photovoltaic cell. Photocurrent characteristics with several external resistances; rectangular cell Model 10A; active area 0.70 sq in. Figures on curves, ohms. (Bradley Laboratories, Inc.)

of measurement of weak beams of radiation to arise from the quantized nature of the radiation itself and from the concomitant statistical fluctuations in radiation intensity (Johnson and Llewellyn, 1934).

The operation of photoemissive cells depends on the release of electrons from a photosensitive surface on incidence of quanta of adequate energy. Since the energy of a quantum is proportional to the frequency of the radiation, there is for any surface a minimum value of frequency—or a maximum value of wave length—below (or above) which the quanta will not have sufficient energy to release electrons. This maximum wave length is known as the threshold wave length for the photosurface in question.

For many metallic surfaces, the threshold wave length lies in the ultraviolet. This circumstance has made possible the design of photocells

which are sensitive only within well-defined spectral regions; the upper wave-length limit is defined by the quantum threshold and the lower by the absorption properties of the photocell envelope.

Thus a cell with a cadmium-magnesium surface and a Corex-D window has a spectral response curve closely paralleling the action spectrum for erythema production, and is of considerable utility in the measurement of the erythema effectiveness of various sources (Koller and Taylor, 1935; Kerr, 1947; Taylor, 1944). Other cells with magnesium (Coblentz and Cashman, 1940), titanium (Coblentz and Stair, 1935; Kuper *et al.*, 1941), or uranium (Rentschler, 1930) surfaces have found use for the measurement of the intensity of extreme ultraviolet radiation from the sun.

Other metals such as zirconium, thorium, tantalum, platinum, and alloys such as beryllium-copper have threshold wave lengths at various places in the ultraviolet spectrum and might be used to provide photocells with specific spectral response characteristics (Rentschler *et al.*, 1932; Glover, 1941; Andrews, 1945; Morrish *et al.*, 1950; Piore *et al.*, 1951). The precise threshold wave length and spectral response curves of these metallic surfaces depend considerably on the particular method of preparation (Déjardin, 1933).

Most metallic surfaces, however, have relatively low quantum efficiency, emitting one electron per  $10^4$ – $10^5$  incident quanta (Sommer, 1947). Hence, most modern photocells are made with composite surfaces, such as the cesium-antimony surface which has a quantum yield of approximately 0.1–0.3 at the wave length of maximum response (400 m $\mu$ ) (Janes and Glover, 1941; Sommer, 1947; Morton, 1949; Zworykin and Ramberg, 1949, Chaps. 5 and 6) and maintain a high yield well into the ultraviolet (Fig. 4-4).

Glass-jacketed photocells begin to decline in sensitivity at wave lengths less than 3500 Å. Commercially available ultraviolet-sensitive photocells have envelopes of Corex-D, Corning 9741, or Vycor glass. The Vycor glass provides good transmission to approximately 210 m $\mu$ , but begins to absorb appreciably at shorter wave lengths (Nordberg, 1947). Some response may be obtained to wave lengths as short as 160 m $\mu$  (Dunkelman and Lock, 1951), owing in part to fluorescence of the glass. Special quartz-jacketed photocells have high sensitivity to 175 m $\mu$  and will respond to wave lengths as short as 155 m $\mu$ .

In the vacuum-type photoemissive cell, the current developed is directly proportional to radiation intensity over several decades of intensity range. The current developed, obtained in a typical photoemissive cell as a function of anode voltage, is shown for several radiation intensities in Fig. 4-5. Evidently at any anode voltage greater than 25 volts, the full photocurrent is collected, and the current is thus substantially independent of anode voltage. The total current which may be drawn from a photo-surface without damage is limited to values of 5–10  $\mu\text{a}/\text{cm}^2$ .



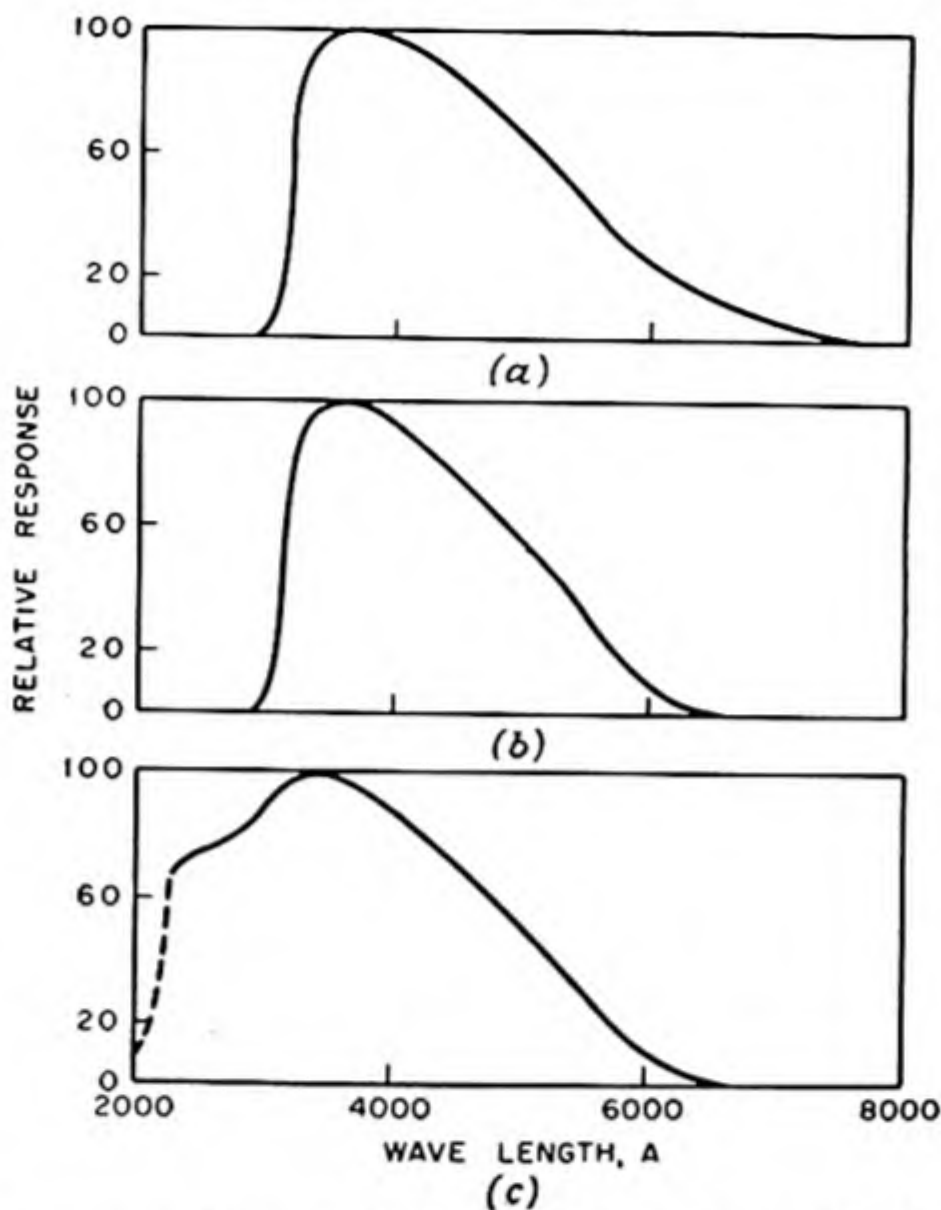


FIG. 4-4. Spectral response characteristics for three types of photosurface: (a) type 1P22, S-8 response; (b) types 931-A and 1P21, S-4 response; (c) type 1P28, S-5 response. (Engstrom, 1947a; *Journal Optical Society of America*.)

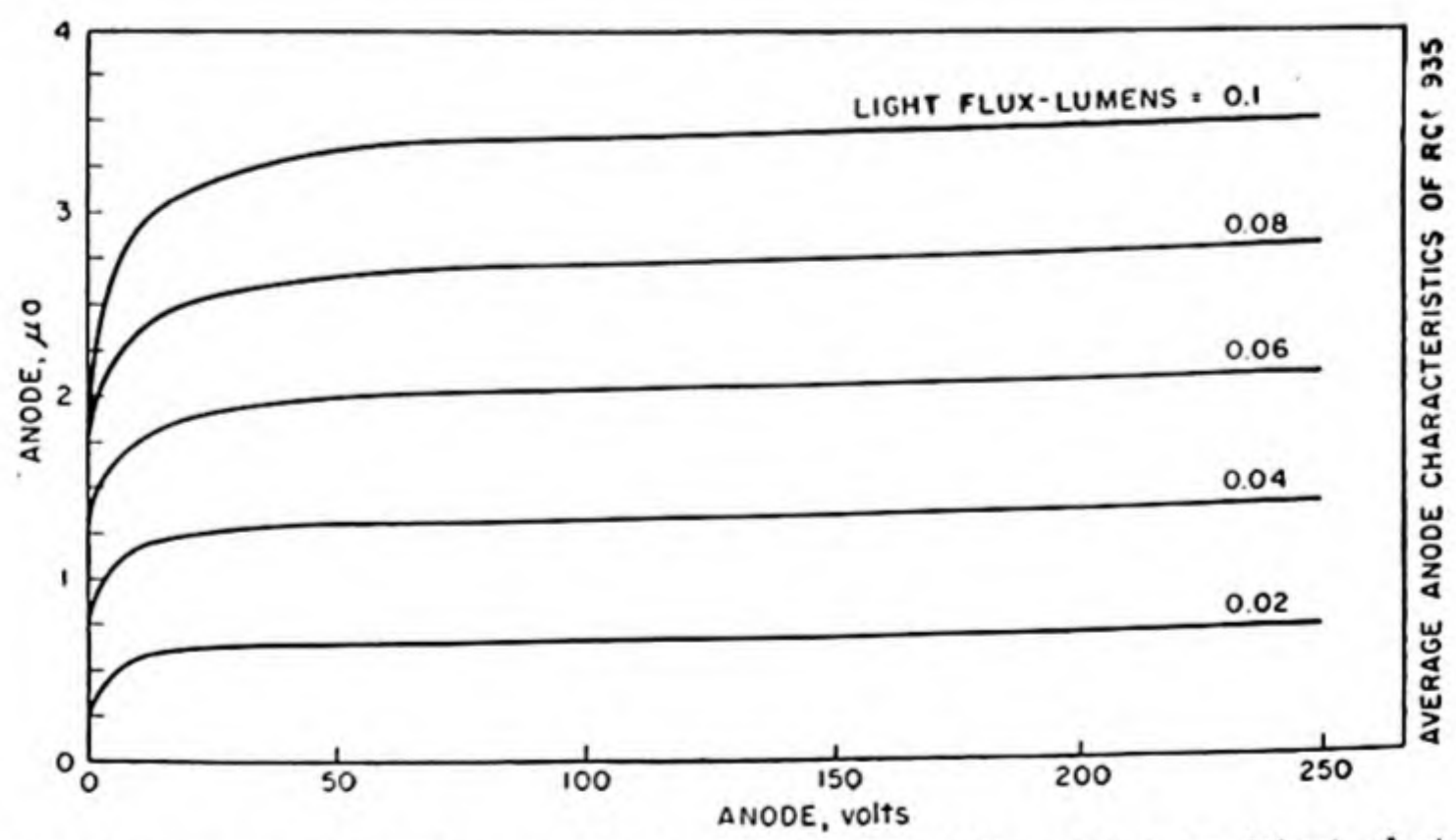


FIG. 4-5. Current-voltage characteristic of RCA 935 (ultraviolet sensitive) photocell. (Radio Corporation of America.)

At low levels of radiation intensity, the photocurrent generated will be small. This current may be amplified external to the cell by conventional vacuum tube circuits (Zworykin and Ramberg, 1949, Chaps. 12-14) or it may be amplified within the cell, either by gas multiplication or by the use of secondary emission, as in the photomultiplier tubes. The

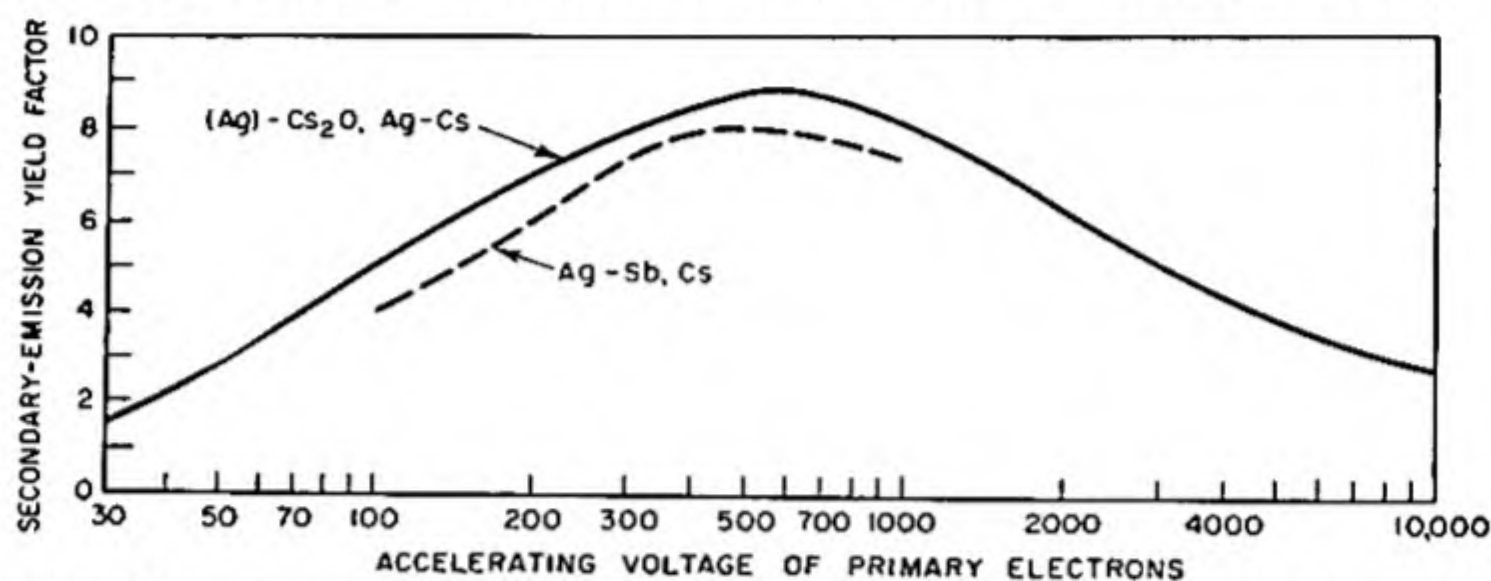


FIG. 4-6. Secondary-emission characteristics of typical photosurface materials. (Reproduction from *Photoelectricity and Its Application*, by V. K. Zworykin and E. G. Ramberg, John Wiley & Sons, Inc., 1949.)

advent of photomultiplier tubes has largely supplanted the use of gas-filled tubes. The direct or amplified photocurrent may be measured with a galvanometer or ammeter, may be recorded, may be integrated in discrete quantities and counted (Douglas, 1947; Launer, 1949), or may be used to operate such devices as relays and motors.

**Photomultiplier Tubes.** In the photomultiplier tubes, the primary current from the photosurface is multiplied by a factor which may be as large as  $10^7$  by repeated use of multiplication at secondary emission surfaces. Many surfaces, including those commonly used as photosurfaces, will, when struck by an electron of appropriate energy, emit several electrons. The number given off per primary electron depends on the surface and the voltage applied to the primary electron (Fig. 4-6) (Zworykin *et al.*, 1936; Morton, 1949).

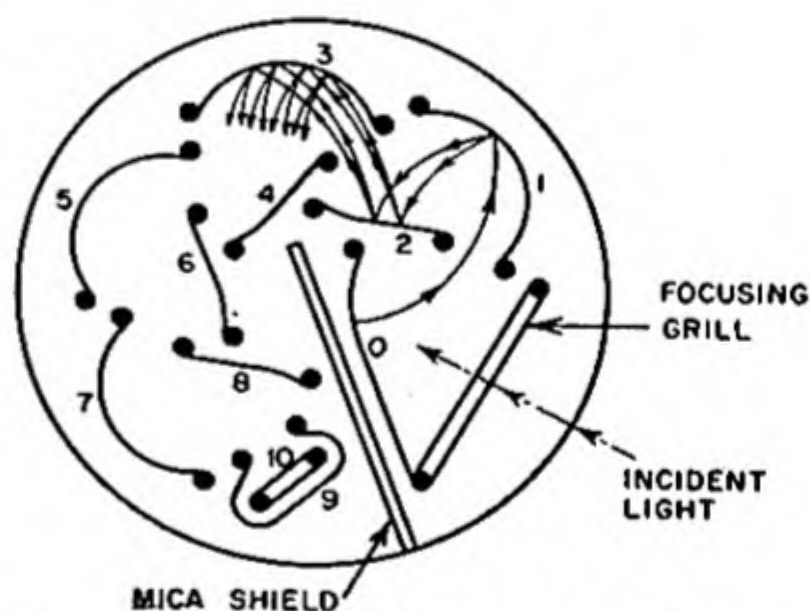


FIG. 4-7. Construction of nine-dynode focused photomultiplier tube. 0, photo-cathode; 10, anode; 1-9, dynodes. (Engstrom, 1947a; *Journal Optical Society of America*.)

In the focused photomultiplier tubes (Rajchman and Snyder, 1940), the primary photocurrent is focused by an electrostatic field onto such a surface, called a dynode. This process is repeated nine or ten times until the vastly amplified current from the last secondary emitting surface is collected on an anode (Fig. 4-7).



An alternative design, such as is used in the "venetian blind" photomultiplier tubes (Sommer and Turk, 1950), does not attempt to focus the electrons from each dynode upon the next, but merely uses an accelerating field to draw the majority of secondary electrons (70–85 per cent) to the succeeding dynode (Fig. 4-8).

Thus a conventional photomultiplier tube consists of a photocathode, a series of 9–11 secondary emission dynodes, the first of which is maintained at a potential 75–150 volts above that of the photocathode while each succeeding dynode is elevated another 75–150 volts in potential in sequence, and a final anode which is maintained 50–100 volts above the potential of the last dynode. With a multiplication of 3–5 per dynode, the over-all amplification of a 9 dynode tube can range from  $3^9$  to  $5^9$  or 20,000 to 2,000,000.

Because the current capacity of the last dynode or anode is limited, there is, at normal gain, a maximum current which may be drawn from the photosurface, and hence a maximum illumination to which it should be exposed. This limit, which will be less than  $1 \mu\text{w}$  for a 1P28 photomultiplier tube operated at a current amplification of  $10^5$ , may be raised if the voltage applied per stage is reduced.

The response of photomultiplier tubes is a linear function of light intensity over many decades. Fatigue is inappreciable at low light levels. Because of the variations in secondary emission with dynode voltage, the voltage supply for the photomultiplier tube potentials

must be held stable to an order of magnitude better than the stability desired in the output current. Batteries may be used, or regulated electronic supplies have been described (Plymale and Hansen, 1950; Higinbotham, 1951; Hill, 1945; Mautner, 1947).

The over-all amplification of the photomultiplier is very closely a logarithmic function of the voltage applied per dynode, over several decades of gain (Fig. 4-9). As a consequence of this circumstance, it can be shown that, for varying levels of illumination, the voltage per dynode necessary to maintain a constant output current is proportional to the logarithm of the reciprocal of the intensity of the illumination. This property may be used in the design of circuits intended to measure absorption directly in terms of optical density (Sweet, 1946).

The time resolution of a photomultiplier tube is limited only by the

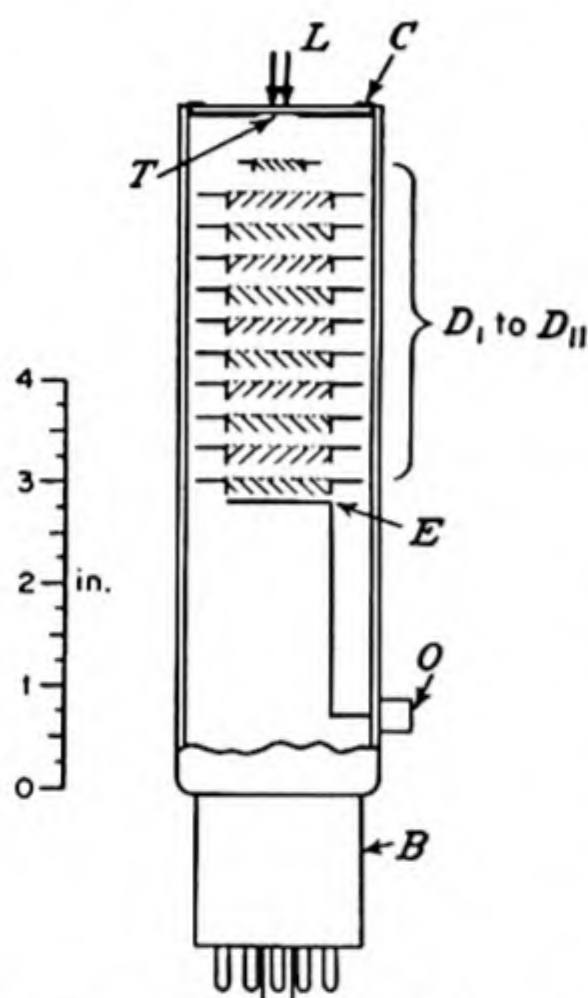


FIG. 4-8. Design of "venetian blind" type photomultiplier tube: *T*, photosensitive surface; *D*, dynodes; *E*, collecting anode. (Sommer and Turk, 1950; *Journal of Scientific Instruments*.)

variations in time of transit of electrons from photosurface to anode, which are of the order of  $6 \times 10^{-9}$  sec (Morton, 1949). As a consequence, the photomultiplier tube will faithfully respond to very brief pulses of light, as short as  $10^{-8}$  sec.

The amplified photocurrent from the photomultiplier tube easily overwhelms the random fluctuations in electric current arising in the external circuit, as a result of thermal agitation, so that the only limitations on the sensitivity of a photomultiplier detector are those arising from the random fluctuation of the "dark current" which is actually the thermal emission of electrons from the photosurface, and from those inherent in

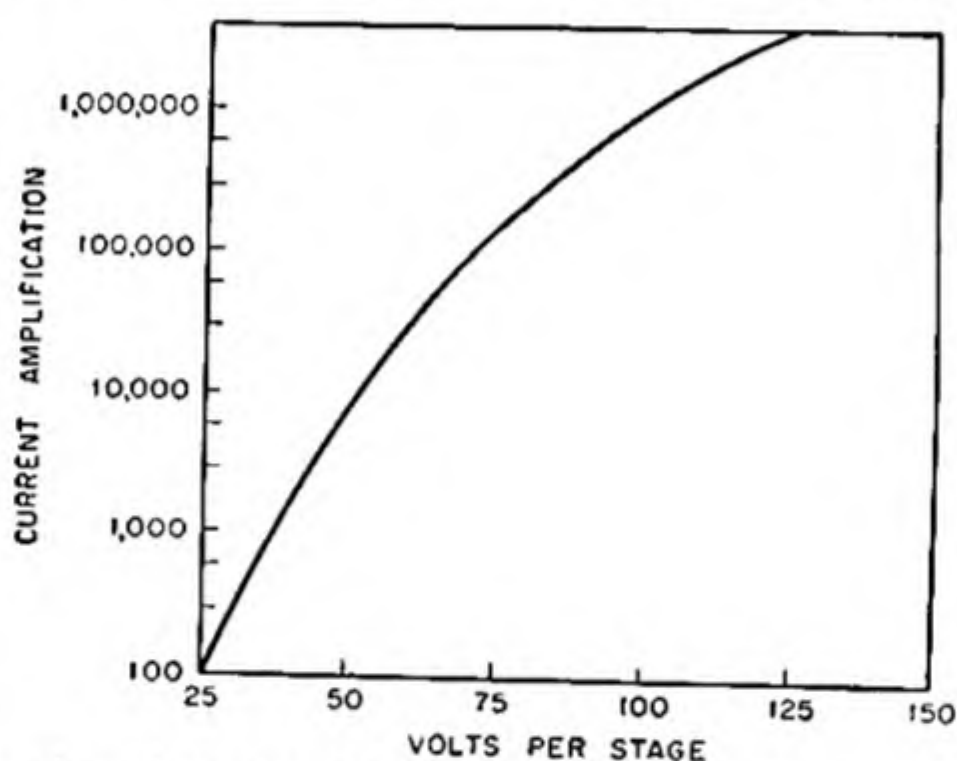


FIG. 4-9. Amplification characteristic of focused type photomultiplier. (Engstrom, 1947a; *Journal Optical Society of America*.)

the statistical nature of the radiation intensity itself. The thermal-emission dark current and its corresponding fluctuations may be reduced by choice of a photocell with a small photosensitive surface, or it may be minimized by refrigeration of the photocell, without appreciably influencing the sensitivity to radiation (Engstrom, 1947a, b).

It should be recognized that photoelectric detectors vary considerably from tube to tube (of the same design) with regard to sensitivity, to variation of sensitivity with wave length, and to the dark current. Because of these variations, if it is desired to use photoelectric detectors for the comparison of two beams of radiation, one of two courses is necessary: (1) some artifice whereby one detector may be used must be employed, or (2) if two detectors are used, either matched tubes must be found, or some means of compensating for their differences (which may be expected to be reasonably stable) must be provided. A single detector may be used, if it is alternately exposed to the two beams in time, or if the electrical signal arising from each beam can be distinguished by virtue of a frequency or phase modulation (Wright and Herscher, 1947; Savitzky and Halford, 1950; Wyckoff, 1952).



The use of modulated radiation, giving rise to an oscillatory current, permits the use of alternating-current amplifiers and thus simplifies the associated electronic circuitry. Alternatively, a steady photocurrent may be converted to an oscillatory current, either by magnetic modulation (Kalmus and Striker, 1948) of the photocurrent, or by mechanical interruption (Liston *et al.*, 1946; Lash, 1949).

*Image Orthicon.* The application of television techniques (Zworykin and Ramberg, 1949, Chaps. 16, 17) to ultraviolet spectroscopy through the medium of ultraviolet-sensitive image orthicon tubes has significant potentialities. The orthicon is in effect a two-dimensional photoelectric detector which permits the application of photoelectric techniques to problems that previously could be adequately approached only by photographic means. The use of the image orthicon by Parpart and Flory for the visualization of ultraviolet microscope images (Parpart, 1950; Flory, 1951), and the study of source spectral characteristics (Benn *et al.*, 1949; Agnew *et al.*, 1949) may be cited as examples of the potential applications.

### METHODS OF SPECTRAL ISOLATION

One of the important data in any problem in radiation biology is the variation of the subject under investigation (absorption, fluorescence, photobiological or photochemical effect) with the wave length of the radiation concerned. To obtain these data, spectrally defined beams of radiation must be available. A wide variety of devices have been developed to provide such spectrally defined beams; these devices differ in basic principles and in range of application and, in general, may have specific advantages or disadvantages for a particular application. For the isolation of well-separated spectrum lines from a discontinuous source, much simpler techniques can be employed than are necessary to isolate narrow spectral band widths from a source of spectral continuum. For some purposes, high intensity or large total energy of radiation are more important than purity of wave length. For others, flexibility and the possibility of easy, rapid change of wave length are important. The optimum means of spectral isolation can be chosen only after the research objectives are clearly defined.

The various means employed for spectral isolation may be somewhat arbitrarily grouped into two classes: filters, which by one means or another block or prevent transmission of all save the selected band of wave lengths, and dispersing systems, which transmit all wave lengths, but disperse them in space so that particular regions may be selected.

#### FILTERS

*Absorption Filters.* The simplest filters are absorption filters. By virtue of the absorption spectra of their components these filters absorb,

TABLE 4-2. ULTRAVIOLET FILTERS

Band pass, transmission	Reference
Wave-length region transmitted, $m\mu$ :	
190-290	Heidt, 1939
230-265	Bäckström, 1940
230-330	Bäckström, 1940; Mazza, 1940
230-420	Corning, 1948
240-280; >350	Kasha, 1948
245-275	Kasha, 1948
245-290; >340	Kasha, 1948
255-290	Kasha, 1948
270-325	Dorcas and Forbes, 1927
290-340	Kasha, 1948
295-330	Bäckström, 1940
300-340	Kasha, 1948
300-400	Corning, 1948; Schott-Jena, 1952
320-360	Kasha, 1948
320-390	Corning, 1948
340-390	Kasha, 1948
For specific spectral lines, $m\mu$ :	
Hg 254	West, 1946
Hg 254 + 265	Bowen, 1946
Hg 280	Bäckström, 1940; Bücher and Kaspers, 1946
Hg 313	Bäckström, 1940; Bowen, 1946; Bücher and Kaspers, 1946; Hunt and Davis, 1947; West, 1946
Hg 334	Bowen, 1946; Bücher and Kaspers, 1946
Hg 366	Bowen, 1946; Bücher and Kaspers, 1946; Corning, 1948; West, 1946
Cd 326	Bowen, 1946
Band pass, absorption	
Wave-length region absorbed, $m\mu$ :	
280-390 ( $Cl_2$ )	Gibson and Bayliss, 1933; von Halban and Siedentopf, 1922
350-540 ( $Br_2$ )	Acton <i>et al.</i> , 1936
290-360 ( $CS_2$ )	Bowen, 1946
340-800	Bäckström, 1940
Long-wave pass	
Approximate cut-off wave length, $m\mu$ :	
190-200	Haas, 1935
210-230	Macleane <i>et al.</i> , 1945
220-260	Corning, 1948
230-250	Bass, 1948; McLaren and Pearson, 1949
245-260	Bass, 1948
260-280	Kasha, 1948
260-310	Corning, 1948
265-275	Bass, 1948; Maclean <i>et al.</i> , 1945
270-280	Bass, 1948; Bowen, 1946; Maclean <i>et al.</i> , 1945



TABLE 4-2. ULTRAVIOLET FILTERS.—(Continued)

Long-wave pass	Reference
280–300	Bass, 1948; Bowen, 1946; Maclean <i>et al.</i> , 1945
280–320	Corning, 1948; Schott-Jena, 1952
290–310	Polaroid, 1951
300–310	Kasha, 1948; Ley and Wingchen, 1934; Saunders, 1928
300–330	Corning, 1948; Schott-Jena, 1952
310–330	Kasha, 1948; Polaroid, 1951; Schott-Jena, 1952
315–365	Maclean <i>et al.</i> , 1945; Schott-Jena, 1952
340–360	Kasha, 1948; Schott-Jena, 1952
340–380	Corning, 1948; Schott-Jena, 1952
350–380	Polaroid, 1951
360–400	Bowen, 1946; Corning, 1948; Schott-Jena, 1952
365–430	Corning, 1948; Schott-Jena, 1952
380–410	Eisenbrand and von Halban, 1930; Polaroid, 1951
420	Bowen, 1946

more or less strongly, all wave lengths other than those of the selected region. Such filters are simple to use, may be made in large dimensions, and place no limitations on the angular spread of the radiation to be transmitted. On the other hand, it is difficult to obtain absorption filters which can provide both a narrow transmission band and high transmission within the band; further the design of an absorption filter for any particular spectral region is a wholly empirical enterprise.

Absorption filters may be made of glass, of liquid cells, or of gas-filled cells (chlorine and bromine) or combinations of these. In general, a filter need not (and will not) transmit only a narrow band of wave lengths out of the entire electromagnetic spectrum. Consideration must be given to the characteristics of the radiation source and the radiation detector, or biological subject, to be employed. Transmission bands in far-removed wave-length regions, such as the infrared, might well be of no consequence in particular investigations.

References to band-pass and long-wave-length-pass absorption filters for various regions of the ultraviolet are summarized in Table 4-2. Appropriate combinations of these may be employed for isolation of particular spectral lines from various sources.

If the absorption of the ultraviolet radiation involves a photochemical decomposition of some component of the filter, the filter may have to be renewed frequently. This is particularly likely with liquid filters including organic components. Such decomposition may sometimes be minimized by placing the sensitive component farthest from the source in the sequence of filter elements.

**Interference Filter.** The action of an interference filter is based on the cancellation of coherent light waves when they are added together in phase opposition. In its action, each ray of the light beam is divided into a large number of weaker rays, with regular shifts in phase between adjacent rays, by the use of multiple reflections between lightly silvered surfaces appropriately spaced by dielectric, these surfaces allowing a slight transmission of energy at each contact (Fig. 4-10). When these rays are recombined by a lens, the resultant intensity at the focus of the lens will depend on the phase difference between adjacent rays, being maximal when all rays are in phase, and minimal when adjacent rays are exactly out of phase. If a stop is then placed about the focus of the lens,

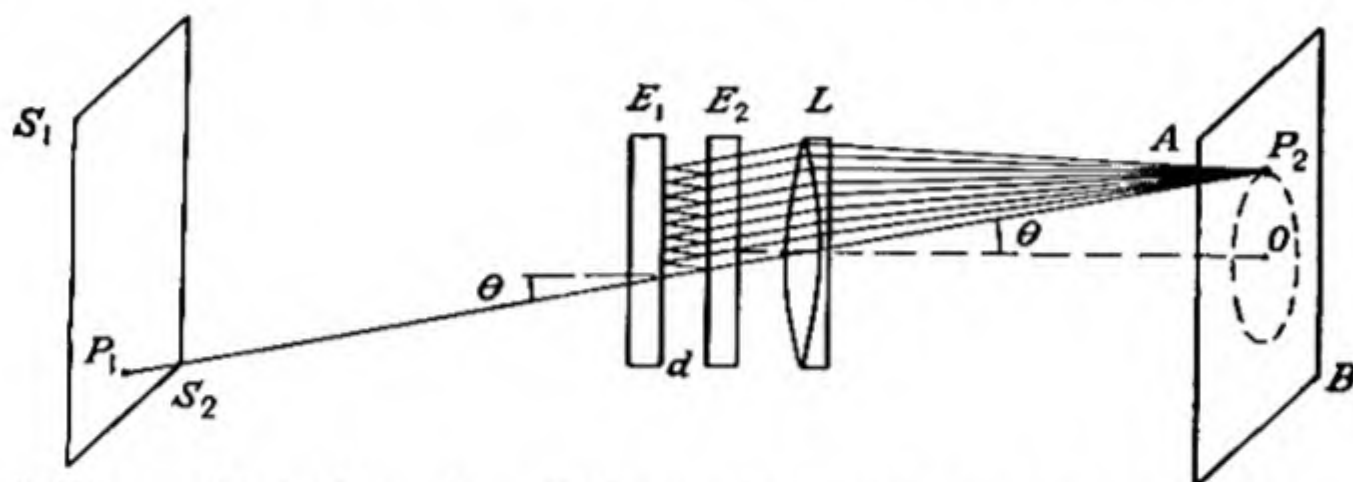


FIG. 4-10. Figure illustrating the splitting and multiple reflection of light rays originating from  $P_1$  by the silvered surfaces  $E_1$  and  $E_2$ , to provide a monochromatic image of  $P_1$  at  $P_2$ . (Reproduction from *Fundamentals of Optics*, 2d ed., by F. A. Jenkins and H. E. White, McGraw-Hill Book Company, Inc., 1950.)

only those wave lengths for which adjacent rays are exactly or nearly in phase will be transmitted in appreciable intensity. By controlling the thickness of dielectric between the reflecting surfaces, the variation of phase difference between adjacent rays with wave length can be controlled and thus the wave length or wave lengths of maximum transmission selected. The simple interference filter can be regarded essentially as a crude Fabry-Perot etalon (Jenkins and White, 1950, Chap. 14).

Thus a simple interference filter consists of two lightly silvered reflecting surfaces, spaced by an appropriate thickness of dielectric (frequently magnesium fluoride). The spectral selectivity of such a filter is dependent extrinsically on the angular aperture of the radiation with which it is employed (increasing with decreasing aperture), and intrinsically on the reflectivity of the reflecting surfaces and the number of wave length paths in the dielectric spacer (Mooney, 1946; Hadley and Dennison, 1947, 1948). Under favorable optical conditions, such filters can provide a peak transmission of about 35 per cent with a band width of about 100 Å (at half-maximum transmission) when peaked for various wave lengths in the visible region. The transmission of interference filters does not, however, drop to zero outside the transmission band (or bands) but to a minimum of about 1 per cent. The wave length of peak transmission



is specified on the assumption that the filter will be used with radiation at normal incidence (Buc and Stearns, 1950).

More complex "multilayer" interference filters (Banning, 1947b; Polster, 1949, 1952), which rely on the cancellation of rays multiply reflected between sandwiches of dielectric layers of appropriate thickness and refractive index (replacing the silvered surfaces), can provide higher transmission (70–80 per cent) and narrower band widths (50–60 Å) at half-maximum transmission).

The simple interference filters cannot be satisfactorily made for wave lengths less than 3600 Å because of the decline in the reflectivity of silver. Aluminum reflectors have not proved satisfactory. It would seem possible to extend the range of the multiple layer dielectric filters farther into the ultraviolet if dielectrics combining proper refractive indices and ultraviolet transparency can be found.

*Christiansen Filters.* If rough chips of transparent dielectric are suspended in a cell containing a transparent liquid, the resultant mass will be highly scattering and hence of low transmission, except at or near the wave length at which the refractive index of the liquid matches that of the dielectric. When employed with an appropriate optical system, such a cell constitutes a Christiansen filter (Christiansen, 1884).

Such filters can be made with large cross section. Their spectral selectivity depends inversely on the angular divergence of the radiation passing through them, directly on the difference in the slopes of the refractive index versus wave-length curves of the liquid and solid at their point of intersection (Raman, 1949) (the curve for the liquid always has the greater slope), directly on the thickness of the cell, and also on the size of the dielectric chips, for which there appears to be an optimum (Denmark and Cady, 1935). As the refractive indices of liquid and dielectric generally vary at different rates with temperature, the wave length of peak transmission of Christiansen filters is strongly temperature dependent.

Appropriate dielectric and liquid mixtures have been described for the visible region by MacAlister (1935), for the 3100–4000 Å region by Kohn and von Fragstein (1932), and for the 2300–3100 Å region by Sinsheimer and Loofbourow (1947). A filter for the mercury 2537 Å line has been described by Minkoff and Gaydon (1946); von Fragstein (1938) mentions filters centered at 2610 and 2450 Å. It should be emphasized that the transmission and spectral selectivity of these filters depend strongly on the optical system in which they are employed (Weigert and Staude, 1927; von Fragstein, 1938). The transmission of Christiansen filters does not decline to zero outside the transmission band but to a minimum dependent on the optical system employed.

*Focal Isolation Filters.* The focal length of a simple uncorrected lens depends on its refractive index and hence on the wave length of the radiation. At the focal plane of any given wave length, radiation of other

wave lengths is necessarily not in focus and is spread out more or less diffusely. If the image of a small source is sharply masked at the focal plane of the wave length desired, this wave length will be favored in the radiation transmitted. If this process is repeated several times (the same lens may be used in autocollimating schemes), quite narrow spectral band widths may be obtained. Such a device is known as a focal isolation filter.

The selectivity of such a filter will depend on the dispersive power of the lens material, on the number of lenses employed, and on the size of source and angular aperture of transmitted radiation. Fluorite or quartz lenses have been employed in this fashion to isolate spectrum lines in the vacuum ultraviolet (Forbes *et al.*, 1934; Duncan, 1940).

*Miscellaneous Filters.* A simple ultraviolet filter which can be used in well-collimated light to reject all wave lengths greater than an arbitrarily chosen boundary, has been described by Regener (1936). A thin film of paraffin oil is sandwiched between the long sides of two  $90^\circ$  quartz prisms. Since the refractive index of the paraffin oil is less than that of the quartz, total internal reflection can occur at the first oil-quartz interface; owing to the greater refractive dispersion of the paraffin oil, there will be, for any angle between the entrant beam and the oil-quartz interface, some critical wave length above which all wave lengths will undergo internal reflection. This critical wave length can be varied by rotation of the interface. Transmission is not complete at wave lengths immediately less than the critical wave length, but increases rapidly with decreasing wave lengths.

Various types of light filters have found employment for special purposes in the visible portion of the spectrum, and could undoubtedly be adapted for use in the ultraviolet, but for one reason or another have not been so used. Among these might be mentioned the polarization interference filter and the rotary dispersion filter.

The former is based on the interference between two orthogonal components of a beam of polarized light after passage through a birefringent crystal; the retardation (in wave lengths) of the one component relative to the other will depend on the thickness and birefringence of the crystal and on the actual wave length, and hence varies with wave length, producing maxima and minima of transmission throughout the spectrum, as the interfering waves combine constructively or destructively (Billings, 1947; Evans, 1949a, b). Filters of this type, using cascaded birefringent elements of appropriate sequence of thickness, have been made with a band width of 1 Å at half-maximum transmission (Billings *et al.*, 1951). Such filters are designed for use at a particular wave length.

Rotary dispersion filters rely for their action on the variation in rotary power of an optically active material, such as quartz, with wave length. A piece of such material, placed between similarly oriented polarizing



elements, will transmit completely only those wave lengths for which the total rotation of the plane of polarization is an integral multiple of  $180^\circ$ , and will reject completely those wave lengths for which the total rotation is an odd multiple of  $90^\circ$ . By cascading a few such elements of appropriately chosen sequence of thickness, an over-all transmission band width of 100–150 Å at half-maximum transmission may be obtained. The wave length of maximum transmission may be varied over a considerable spectral region by a programmed rotation of the various polarizing elements (Cambridge Thermionic Corp., 1952).

This extension of the application of such filters to wave lengths as short as 3000 Å would be straightforward, since Polaroid will transmit well to such wave lengths (Barer, 1949). Below 3000 Å it would be necessary to use prism polarizing elements of limited aperture.

#### DISPERSING SYSTEMS

*Prism Instruments.* Because of the variation of its refractive index with wave length, a prism will deviate rays of different wave length through different angles. If the angular spread of the radiation incident on the prism is limited (by means of an entrance slit, or equivalent, and collimating lens), the radiation emergent in any given direction will contain a limited range of wave lengths. The emergent radiation may be focused by a telescope lens to form a spectrum consisting of a continuous series of images of the entrance slit in light of successively increasing wave length.

The width of the spectral band contained in any one image of the entrance slit will depend on the angular divergence of the radiation incident on the prism, the rate of change of angular deviation produced by the prism with wave length, and the physical breadth (in wave lengths) of the beam emergent from the prism. The first quantity is determined by the entrance slit width and the collimator focal length. The second quantity depends on the dispersive power ( $dn/d\lambda$ ) of the prism material and the length of the base of the prism. The breadth of the emergent beam determines the size of the diffraction disc to which it is focused.

By the use of an appropriately placed exit slit, any portion of the emergent spectrum may be selected. The width of slit used can control the spectral width of the radiation band transmitted, except that it is inefficient to reduce the band width to less than the spread of wave lengths contained in any single image of the entrance slit.

In general, the band width, at half-maximum transmission, of radiation emergent from a monochromator used with symmetrical entrance and exit slits and similar collimator and telescope lenses, is given by the formula

$$\Delta\lambda = \frac{W}{F \times d\theta/d\lambda}$$

where  $W$  = width of exit slit

$F$  = focal length of telescope lens

$d\theta/d\lambda$  = angular dispersion of prism,

or by

$$\Delta\lambda = \frac{W}{f_n \times t \, dn/d\lambda}$$

where  $f_n$  =  $f$ -number of telescope lens

$t$  = thickness of prism base

$dn/d\lambda$  = dispersive power of prism.

The quantity of radiant energy transmitted through the dispersing system depends intrinsically on the size of the entrance slit, the angular aperture of the collimating lens, the height of the prism, and the size of the exit slit. Increased energy may be obtained at the cost of spectral purity by the use of wider entrance and exit slits.<sup>2</sup>

In the ultraviolet region, 2000–4000 Å, quartz (crystal or fused) is the most commonly used prism material. Its dispersion increases rapidly with decreasing wave length, which results in a corresponding increase in spectroscopic resolving power in quartz prism instruments. Crystal quartz of good quality may be used in prisms to wave lengths as short as 1850 Å and in windows to about 1600 Å (Powell, 1934b; Terrien, 1936; Boyce, 1941; Gilles *et al.*, 1949).

Liquid-filled prisms may also be used in the ultraviolet (Forsythe, 1937, pp. 88–89; Cannon and Rice, 1942), although these are temperature sensitive and subject to such difficulties as convection currents due to excess heating of the liquid near the entrant surface. Harrison (1934a) has described some ingenious dispersing systems, employing water as the dispersing element, in which convection is minimized by the use of the upper surface of a water trough as the first surface of the dispersing element. Such a device can be cheaply made in almost any desired size. The dispersive power of water is within 50 per cent that of quartz.

Below 2000 Å, prisms of calcium or lithium fluoride may be used to wave lengths of approximately 1300 and 1200 Å, respectively (Powell, 1934a; Schneider, 1934, 1936, 1937; Kremers, 1940; Boyce, 1941; Stockbarger, 1949).

If uncorrected quartz lenses are used as collimator or telescope, these must be refocused for different wave lengths. Achromatic quartz-lithium fluoride pairs have been developed which are adequately corrected throughout the ultraviolet and visible regions (Perry, 1932; Stock-

<sup>2</sup> An ingenious means for increasing the radiant flux transmitted through the dispersing system, while retaining high spectral purity, has been proposed by Shurcliff (1949). In this proposal, which necessitates the use of two monochromators in tandem, adroitly spaced, multiple entrance slits are employed, thus markedly increasing the light input to the first monochromator. Appropriately placed secondary slits in the spectrum plane between the two monochromators, and exit slits in the final spectrum, then serve to exclude all save the desired wave-length region which is obtained in high energy with essentially double monochromator purity.



barger and Cartwright, 1939; Cartwright, 1939). Mirror optics may also be employed to solve the problem of achromatization.

Multiple prism cascades may be used effectively to obtain greater prism base and hence greater resolving power. Double monochromators, employing essentially two single monochromators in tandem, may be used for greater purity of radiation and freedom from scattered radiation at the

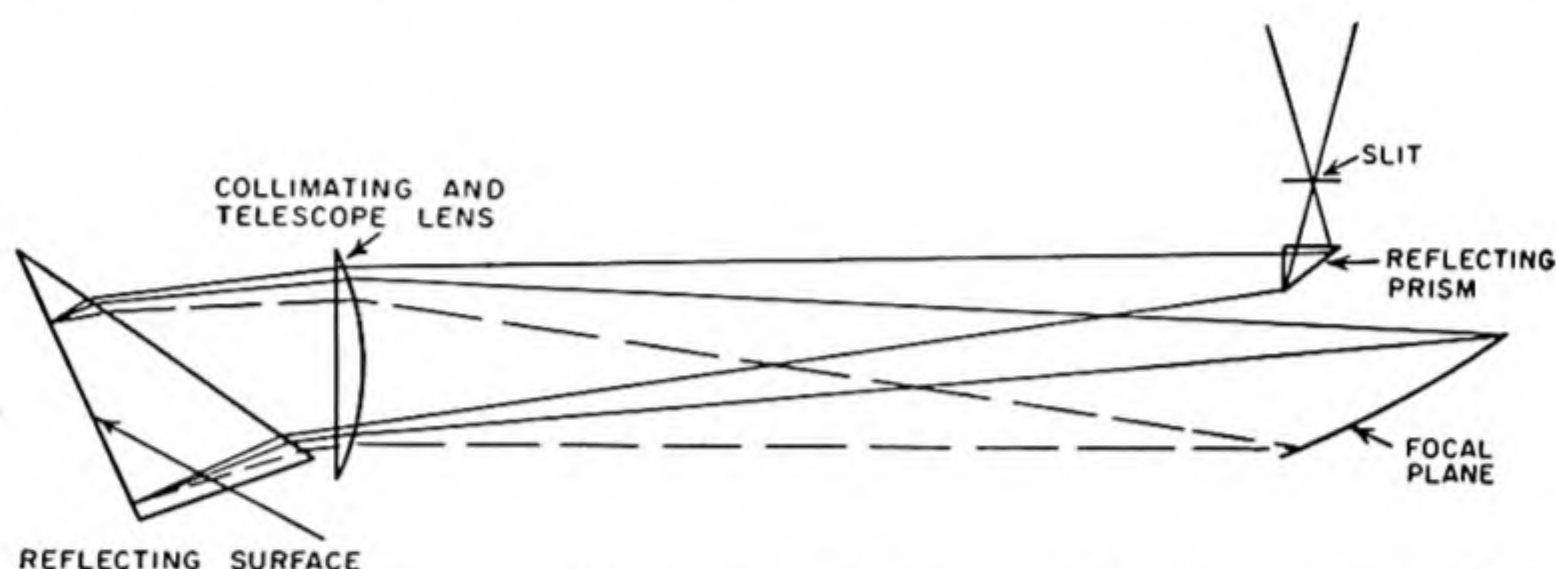


FIG. 4-11. Littrow mounting for prism instrument. (Reproduction from *Practical Spectroscopy*, by G. Harrison, R. Lord, and J. R. Loofbrourow, Prentice-Hall, Inc., 1948.)

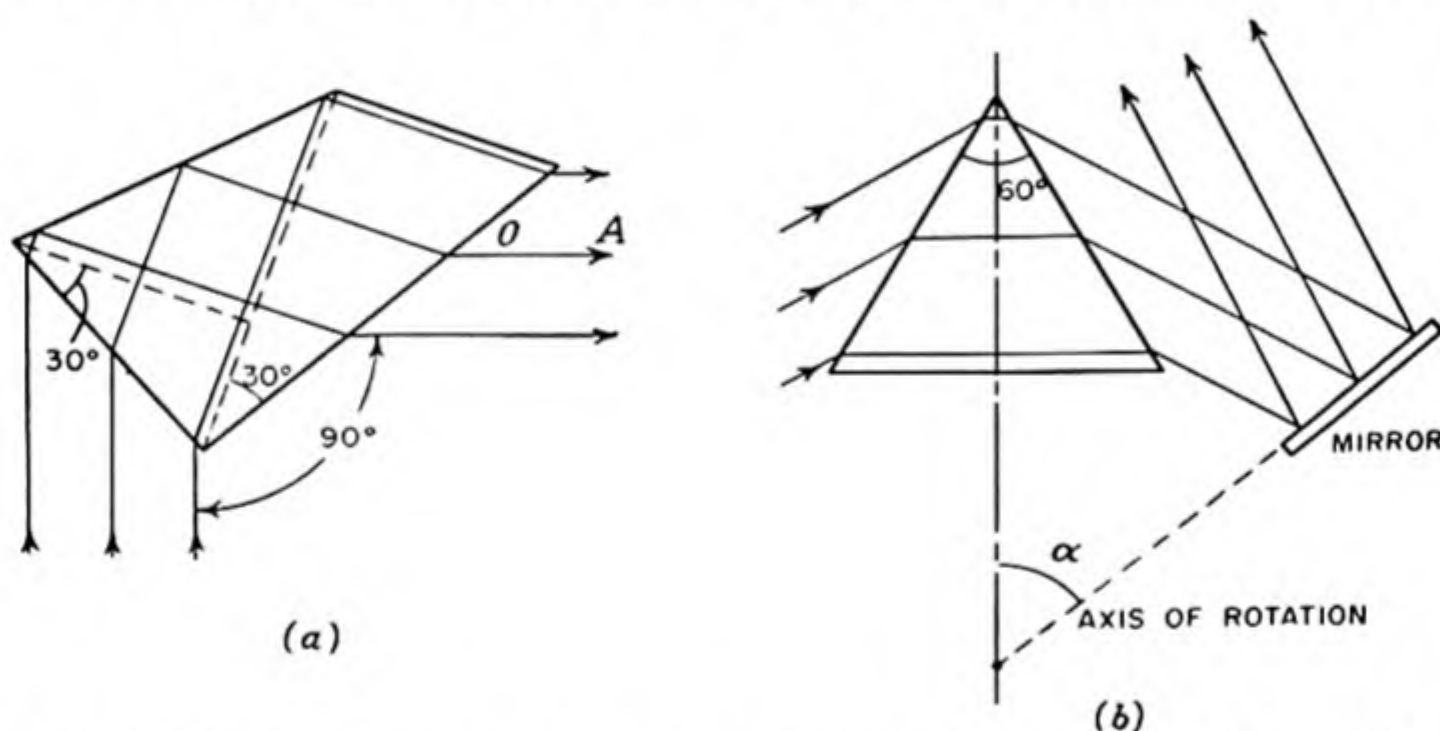


FIG. 4-12. Constant deviation prism instruments using: (a) Pellin-Broca prism; (b) Wadsworth mounting for prism. (Reproduction from *Practical Spectroscopy*, by G. Harrison, R. Lord, and J. R. Loofbrourow, Prentice-Hall, Inc., 1948.)

expense of energy transmission (Sawyer, 1951; Harrison *et al.*, 1948). Cascaded Pellin-Broca prisms may also be employed in such a way as to minimize stray radiation (Benford, 1936).

By reflecting the radiation back through the prism, as in the Littrow mounting (Fig. 4-11), twice the dispersion and resolving power may be obtained. The collimating lens then may serve also as telescope lens. With this arrangement the exit slit is spatially near to the entrance slit, and scattered radiation may be a problem.

Constant deviation monochromators may be made with the Pellin-Broca prism (Fig. 4-12a), with the Wadsworth mounting for the ordinary

prism (Fig. 4-12b), with the Young and Thollon split-prism arrangement (Kurtz, 1926), or with an adroit mirror arrangement recently described by Makishima *et al.* (1951). In these instruments, the entrance and exit slits (and hence the source and monochromatic image) are maintained constant in position while the dispersing element(s) is rotated to vary the emergent wave length.

*Grating Instruments.* If a wave front of radiation is broken into a number of narrow, parallel zones evenly spaced by appropriate distances, the waves propagating from each zone will interfere with those from all other zones so as to produce a diffraction pattern. For any given wave length, there will be some direction or directions in which the waves from each zone will all be in phase to produce a maximum of intensity. In another direction, waves of another wave length will be in phase to yield a maximum of intensity, whereas the waves of the first wave length will largely cancel each other. A device to thus disrupt a wave front is known as a diffraction grating. By thus deviating radiation of different wave lengths into different angles, a grating can serve as a dispersing element.

A grating may consist of a large number of thin, parallel slits, in which case it is a transmission grating, or of a similar number of thin, parallel reflecting strips, in which case it is a reflection grating.

If, in a direction of maximum intensity for a given wave length, the waves from one slit (or strip) are exactly one wave length retarded or advanced with respect to those from the two adjacent slits, this direction is referred to as that of the first-order maximum. If the phase difference between waves from two adjacent slits is just two wave lengths, the direction is that of the second-order maximum. In general, directions of maximum intensity will occur whenever

$$n\lambda = d(\sin \alpha + \sin \beta)$$

where  $n$  = order number (an integer)

$\lambda$  = wave length

$d$  = spacing between slits.

(See Fig. 4-13).

For a given wave length, the distribution of intensity in angle about a direction of maximum intensity, will depend ultimately on the ratio of the

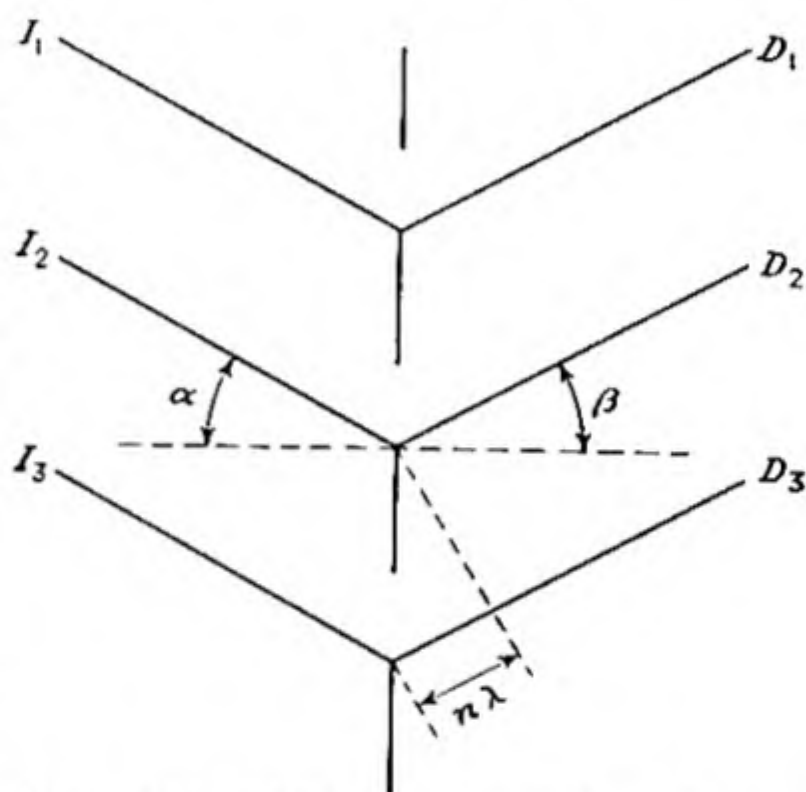


FIG. 4-13. Diffraction by a plane transmission grating.



over-all width of the grating to the wave length, or more specifically on the number of wave lengths of phase difference between the waves, proceeding in a given direction from the extreme slits of the grating. As this phase difference increases, as with increasing order number, the width of the angular intensity distribution decreases, and hence the spectroscopic resolving power increases.

The angular dispersion of a grating—the variation of wave length of maximum intensity with angle—likewise increases with increasing order number, and also with decreasing spacing between the slits. Analytically,

$$\frac{d\beta}{d\lambda} = \frac{n}{d} \cos \beta$$

where  $\beta$  = angle of diffracted beam with grating normal

$n$  = order number

$d$  = spacing between slits.

While both spectral resolution and angular dispersion are favored by the use of higher diffraction orders, the problem of overlapping orders becomes acute for high-order numbers. The third diffraction order of  $\lambda_1$  will overlie the second order of  $1.5\lambda_1$  and the first order of  $3\lambda_1$ , etc. Frequently filters or elementary prism devices may be added to surmount this difficulty and permit the use of second- or third-order spectra.

The grating, transmission or reflection, may be on a plane surface, in which case it is illuminated with parallel light from a collimating lens or mirror, and the emergent beams are focused to a spectrum with a telescope lens or mirror. Or the grating, if reflecting, may be ruled on a concave surface, in which case it will serve as its own focusing element, permitting the elimination of the collimating and telescope elements (Beutler, 1945). Such concave gratings are then effective throughout any wave-length region for which a reflecting surface may be made, since the need for any transparent dielectric is eliminated. The images formed by such gratings are, however, generally astigmatic, unless the grating is illuminated with a parallel beam, as in the Wadsworth mounting (Sawyer, 1951, Chap. 6; Harrison *et al.*, 1948, Chap. 4).

Gratings may be produced with higher resolving power than any prism instrument. By the use of replica techniques, many copies can be made from one master at moderate cost. However, grating instruments necessarily waste light in unused orders, although this drawback can be minimized by proper ruling of the reflecting strips which can serve to direct most of the energy into one order (Wood, 1944; Babcock, 1944; Stamm and Whalen, 1946).

The use of grating dispersing elements in monochromators is a relatively recent development (French *et al.*, 1947), although commercial designs employing plane reflection gratings are now available (Bausch and Lomb, 1951) (see Fig. 4-14). The use of such monochromators may

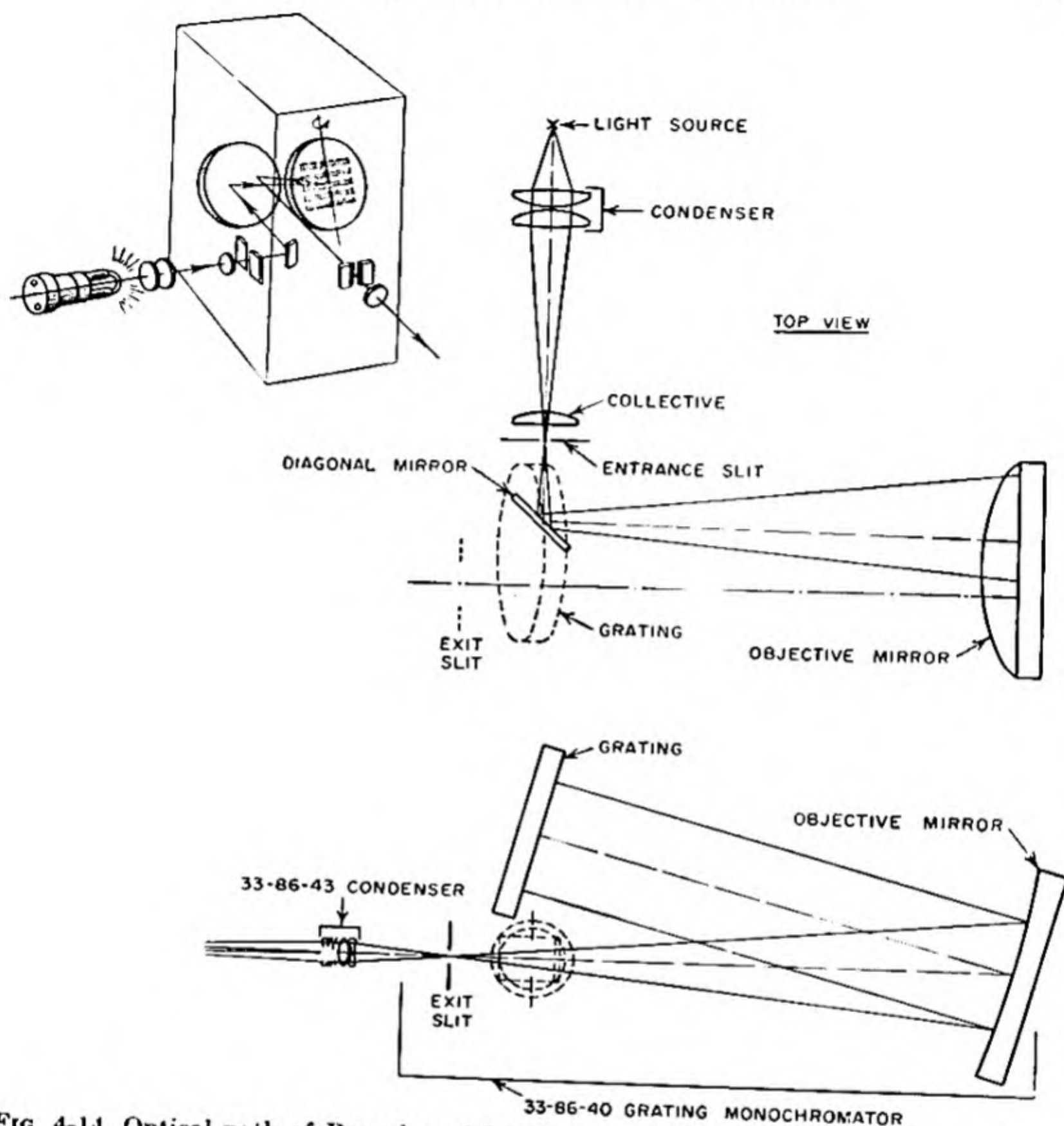


FIG. 4-14. Optical path of Bausch and Lomb grating monochromator. (Bausch and Lomb Optical Company.)

become more widespread as high-quality gratings become more generally available.

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## CHAPTER 5

# Ultraviolet Absorption Spectra

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*Parameters of absorption spectra: Position in the electromagnetic spectrum—Width of absorption band—Intensity. Absorption and chemical constitution: Empirical correlations—Theoretical developments. Specification of absorption: Intensity—Spectral position—Band width. Environmental factors influencing absorption: Solvent—pH value—Concentration—Temperature—Orientation—Scattering—Local concentrations. Ultraviolet absorption spectra of important biological substances: Proteins and amino acids—Nucleic acids and nucleotides—Steroids—Carotenoids—Porphyrins—Flavins—Pterins—Vitamins—Plant pigments. References.*

The absorption of ultraviolet radiation by a molecule results in a change in the electronic configuration of that molecule and therefore in a change, usually transient and reductive, in the stability of the molecule. The ability of any molecule to absorb ultraviolet radiation of a particular frequency is dependent on the electronic configuration of the molecule and the electronic configurations of the possible higher energy states of the molecule. This absorptive ability is thus intimately related to the detailed molecular structure (Lewis and Calvin, 1939; Ferguson, 1948; Bowen, 1946, 1950; Maccoll, 1947). An absorption spectrum of any substance is a quantitative description of the absorptive ability of the molecules of that substance over some range of electromagnetic frequencies.

A knowledge of the absorption spectra of the major components of a living organism makes it possible to limit the number of possible primary receptors of radiation that are found to produce some biological effect. Only those substances that absorb the radiation found to be responsible for the effect need be considered. The correlation of the measured relative efficacy of radiations of various wave lengths in producing the effect, with the variation of absorptive power with wave length of the substances under consideration as primary receptors, can, under favorable circumstances, further limit the possibilities as to the nature of the receptor. A knowledge of the influence of absorption on the stability of the



various substances considered may aid still further in identification of the primary receptor or receptors.

### PARAMETERS OF ABSORPTION SPECTRA

Any absorption spectrum, however complex, may be regarded as a summation of a set of individual absorption bands, each corresponding to a transition between two particular electronic configurations (Fig. 5-1) (Sheppard *et al.*, 1941; Wulf and Deming, 1938). It is usually possible to group these individual bands, each group consisting of transitions involving nearly the same energy difference. The different bands within a group then represent transitions involving a common change in basic electronic configuration, together with varied associated secondary changes in the distribution of energy among the molecular vibrations.

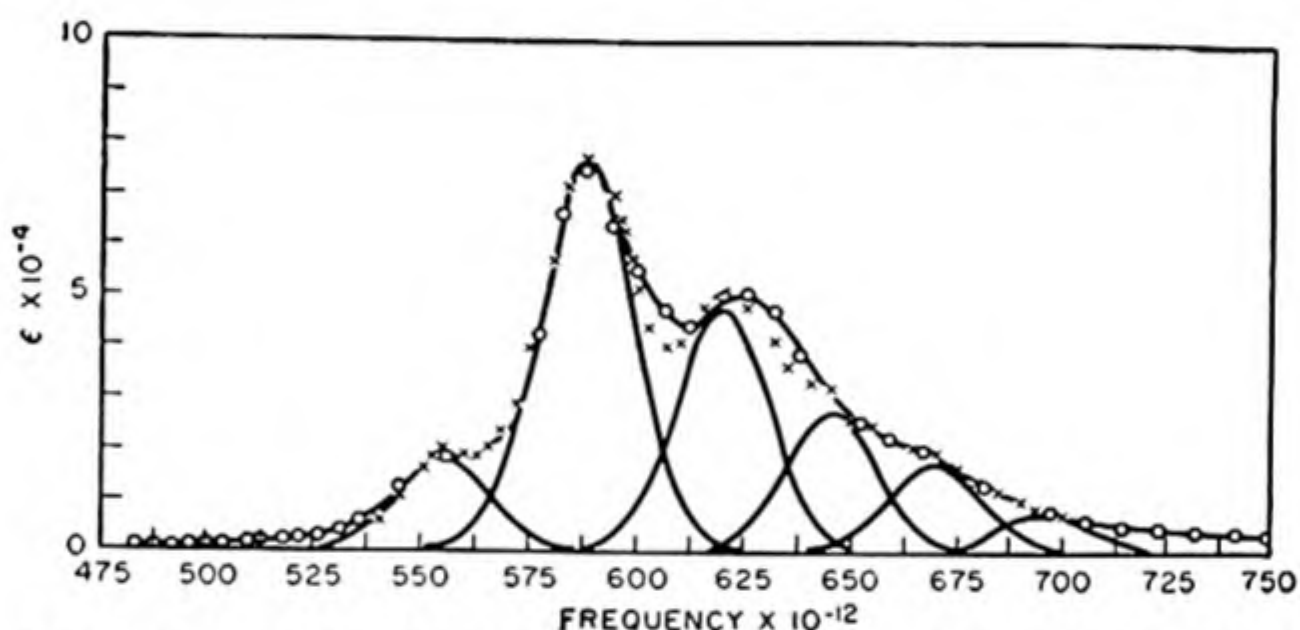


FIG. 5-1. Resolution of the absorption spectrum of merocyanine in hexane into six bands, each representing a particular vibrational transition associated with the fundamental electronic transition. (Sheppard *et al.*, 1941.)

The parameters of an absorption spectrum are properly the sum of the parameters of the individual bands. Any individual band (representing a transition between two distinct electronic states plus vibrational states) may be described by three parameters: (1) the position in the electromagnetic spectrum, (2) the breadth of the electromagnetic spectrum occupied by the band, and (3) the intensity of absorption.

### POSITION IN THE ELECTROMAGNETIC SPECTRUM

The position of an absorption band in the electromagnetic spectrum is dependent on the energy difference between the initial and the excited electronic configurations since this energy difference must be supplied by the absorbed photon, the energy of which is related to its frequency ( $\nu$ ) by Planck's relation

$$E = h\nu = \frac{hc}{\lambda} \quad (5-1)$$

where  $h = 6.61 \times 10^{-27}$  erg-sec

$h = 4.13 \times 10^{-15}$  ev-sec

$c = 3 \times 10^{10}$  cm/sec.

The energy differences corresponding to absorption bands in the ultraviolet region (wave length  $< 4000$  Å) are of the magnitude of 3.1 ev or greater. Since these energies are greater than those that correspond to the energy of formation of many chemical bonds (C—C bond energy = 2.54 ev; C—N bond energy = 2.11 ev) (Pauling, 1945), the rupture of such bonds in molecules raised to an excited level by absorption of an ultraviolet photon is energetically possible. Such rupture may lead to the formation of free radicals or of oppositely charged groups or, in molecules containing atoms with unbonded electron pairs, to photo-oxidation and semiquinone formation (Waters, 1948; Lewis and Lipkin, 1942; Lewis and Bigeleisen, 1943b). The farther into the ultraviolet the absorption band is located, the greater is the excess of excitation energy over the minimum necessary for bond rupture. With absorption bands in the far ultraviolet (wave length  $< 2000$  Å) the absorbed energies generally become adequate to produce molecular ionization (8–12 ev) (Price, 1947).

#### WIDTH OF ABSORPTION BAND

The width of an individual absorption band is dependent intramolecularly on the duration of the excited electronic state (Heitler, 1944, pp. 110ff) and extramolecularly on the statistical distribution of the frequencies of the particular absorption band among the assemblage of absorbing molecules, each exposed to a certain randomness of molecular environment.

Considering any one molecule in a given molecular environment, the width of its absorption band is inversely dependent on the duration (mean lifetime) of the excited electronic state. This may be formulated by the "uncertainty principle"

$$\Delta E \Delta t \approx \frac{h}{2\pi}$$

where, in this instance,  $\Delta E$  is the uncertainty, i.e., variation, in the energy difference accompanying the transition and  $\Delta t$  is the duration of the transition. For the usual absorptive process in an isolated molecule,  $\Delta t$  is of the order of magnitude of  $10^{-8}$  sec,  $\Delta E$  is about  $10^{-7}$  ev,  $\Delta E/E = 10^{-5}$  per cent as is  $\Delta\lambda/\lambda$ .

However, any of several processes may shorten the duration of the excited state ( $\Delta t$ ), thus increasing the uncertainty in energy of the transition ( $\Delta E$ ), and hence may broaden the absorption band. Disruption of the molecule may take place within the duration of a single molecular vibration and thus reduce the excitation lifetime to as short as  $10^{-14}$  sec.



If disruption is less likely, the molecule may remain intact for several hundred or thousand vibrational periods, or  $10^{-10}$ – $10^{-12}$  sec. The spectral broadening in this case will not be so great and will give rise to so-called "predissociation" bands (Rice and Teller, 1949).

The duration of the excited state may be reduced because of a high probability of a transition to a third electronic state (Fig. 5-2). The dura-

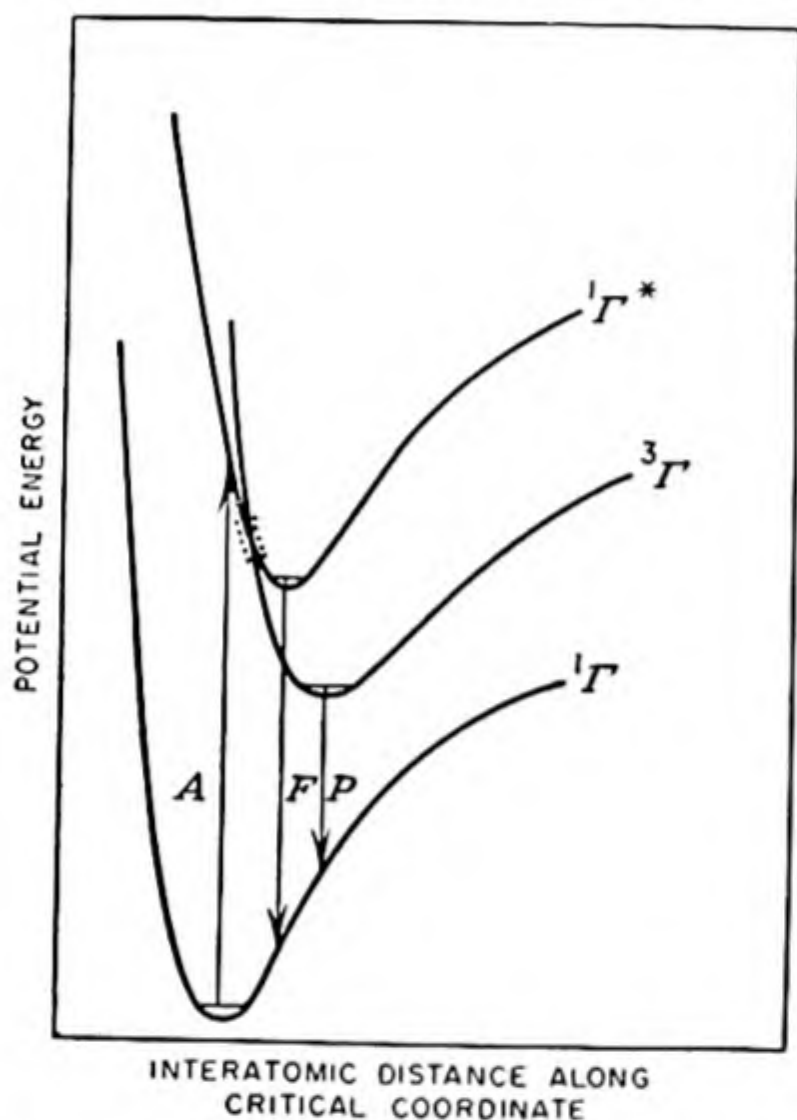


FIG. 5-2. Illustration of the possibility of a radiationless transition from the initial excited electronic state ( $^1\Gamma^*$ ) to a second excited state ( $^3\Gamma$ ), in this instance a triplet state which would have a long duration and from which return to the ground state could occur by delayed emission of radiation or phosphorescence ( $P$ ). The curves represent the variation of potential energy of the molecule as a function of the interatomic separation for a diatomic molecule. (Kasha, 1947; copyright, 1947, by The Williams and Wilkins Company.)

the energy will be reradiated as "fluorescence." This fluorescence radiation may then escape, or it may, under appropriate conditions, be reabsorbed by other chromophores within the solution or biological system (Arnold and Oppenheimer, 1950; Förster, 1948; Franck and Livingston, 1949).

In addition to these primarily intramolecular factors, the electronic

tion of this state will have no influence on the width of the original absorption band. A number of such instances of transitions to a "triplet" state have been reported (Kasha, 1947). Transitions from this triplet state to the original ground state are of very low probability so that the molecule may retain energy as an excited triplet state for appreciable lengths (seconds) of time (McClure, 1949).

Under conditions of appreciable intermolecular contact (solutions and solids) the energy of excitation may be rapidly dissipated by conversion to vibrational energy which, in turn, is simply transferred by collisions or electromagnetic damping to neighboring molecules and ultimately appears as thermal energy (Massey, 1949). Such dissipative effects, which reduce the duration of the excited state, are in part responsible for the broadening of absorption bands of substances in solution as compared to their vapor absorption spectra.

If the molecule retains its excitation energy for a time comparable with the probability of transition from the excited to the ground state,

configuration of the individual molecules and thus the energy associated with a particular electronic transition will be influenced in a condensed system by the electric and magnetic fields associated with nearby molecules. Since the spatial orientations involved will be random (except in crystals) and will be varying, owing to thermal motion, there will result a statistical distribution of electronic configurations and of transition energies of the absorbing molecules, thus broadening the observed absorp-

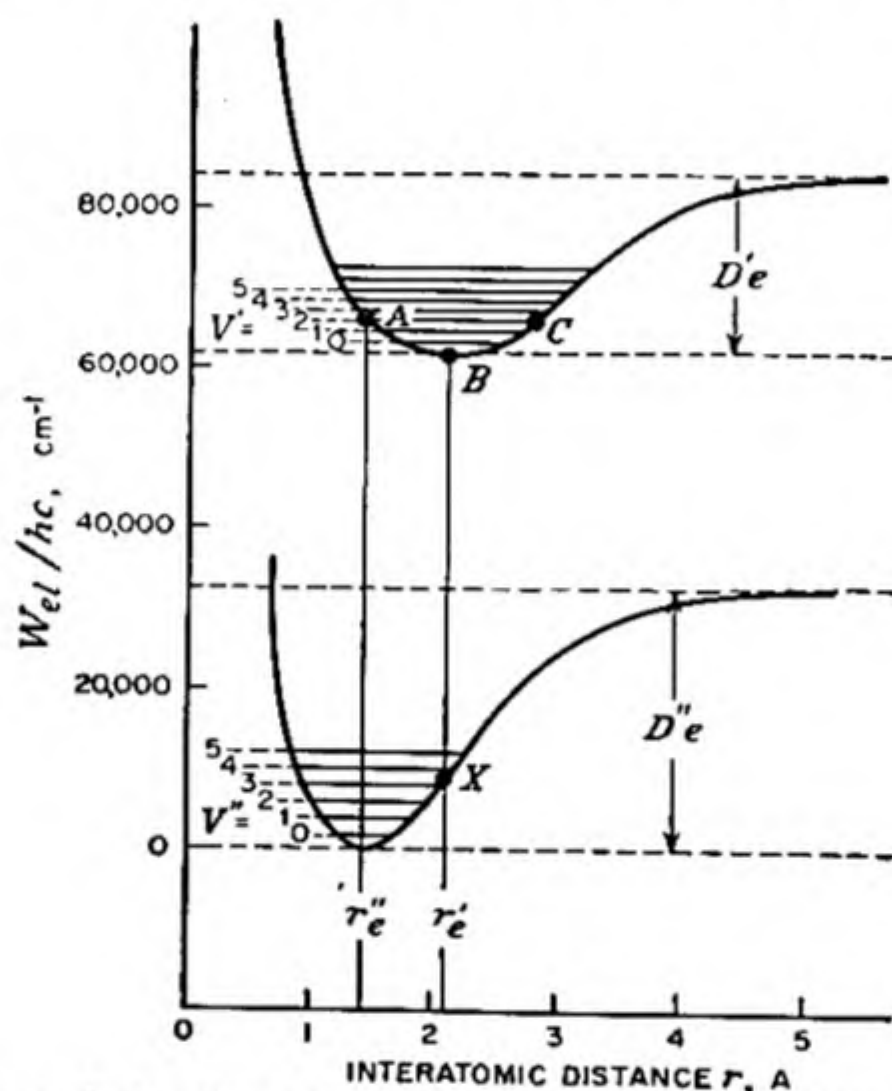


FIG. 5-3. Illustration of the Franck-Condon principle. Horizontal lines within the well of each potential-energy curve represent various vibrational-energy levels. A transition from the ground state ( $V''$ ) to the excited state ( $V'$ ) would most probably leave the molecule in the second excited vibrational level (point A) since the interatomic distance cannot change appreciably within the duration of the transition. (Reproduced by permission of the publishers from *Practical Spectroscopy*, by George R. Harrison, Richard C. Lord, and John R. Loofbrouow, copyright, 1948, by Prentice-Hall, Inc.)

tion band. These effects will be reduced if the fields involved are reduced (as in nonpolar solvents) or if the extent of the variations due to thermal motion is reduced, as in spectra of substances at low temperatures (Sinsheimer *et al.*, 1950a).

For many substances in solution, the effects described widen the individual absorption bands associated with a given electronic transition so as to produce a fusion of these bands into an apparently single band of considerable breadth. The individual bands, representing transitions from vibrational energy states accompanying the normal (lowest energy) electronic state to various vibrational states accompanying the excited



electronic configuration, are thus concealed, reducing the amount of information available in the spectrum. The possible vibrational transitions are limited by the Franck-Condon principle (Rice and Teller, 1949), which simply recognizes that the duration of the electronic transition is brief compared to the duration of a molecular vibration, so that the position of the atoms cannot change appreciably during the act of absorption. Hence only transitions to excited-state vibrational levels, involving atomic configurations similar to those in the vibrational levels associated with the ground state, are probable (Fig. 5-3).

Broadening of this type may also be reduced by a reduction of the temperature of the absorbing substance; the reduction in thermal molecular energy decreases the molecular population in the higher vibrational energy levels and thus reduces the number of possible transitions. Indeed, at liquid-air temperature or below, all molecules must commence a transition from the lowest vibrational energy level.

### INTENSITY

The total intensity of an absorption band, i.e., the integrated absorption over the band, is dependent on the difference in scale and symmetry of the electronic configurations for the initial and the excited states (Heitler, 1944). A net time-average displacement of charge along some molecular axis must accompany the absorption of radiation. If the electronic configurations of the two energy levels are of such a symmetry that a transition from one to the other does not provide such a time-average displacement, then a transition between these levels cannot be induced by radiation, i.e., absorption cannot occur. Such a transition is said to be "forbidden."

In benzene, the electronic configurations of the ground and the first excited singlet energy levels are of such a symmetry that a transition between them is forbidden (Sklar, 1942). This transition, which is associated with the benzene absorption maximum at 2550 Å, can occur only if accompanied by a particular molecular vibration which so distorts the molecule as to alter the symmetry of either the ground or the first excited energy levels and thus gives rise to a small time-average displacement of charge. The intensity of such forbidden absorption bands, which require the participation of a molecular vibration, is generally low. Thus for the benzene absorption maximum at 2550 Å,  $\epsilon = 120$ ; this may be contrasted with the intensity of the "allowed" benzene absorption band at 1835 Å which is about 380 times as great ( $\epsilon = 46,000$ ) (Platt and Kleven, 1947). In general, the greater the time-average displacement of charge, the greater the integrated absorption.

For a given integrated absorption, the intensity of the absorption maximum will obviously depend inversely on the width of the band and thus directly on the duration of the excited state.

## ABSORPTION AND CHEMICAL CONSTITUTION

### EMPIRICAL CORRELATIONS

The empirical correlation of the spectral position of absorption bands in the ultraviolet with certain chemical structures was begun about 1885 with the work of Hartley (1885) and has been steadily continued and expanded as improvements in technique have simplified the task of measuring absorption spectra (Braude, 1945; Lewis and Calvin, 1939; Ferguson, 1948; Brode, 1943; Jones, 1943). The broader long-recognized empirical correlations have now been given a theoretical basis by the development of approximate wave-mechanical methods of calculation of electronic-energy levels in complex molecules.

These correlations early indicated that the absorption bands of compounds, composed exclusively of saturated linkages, occurred generally below 2000 Å, usually in the vacuum ultraviolet below 1850 Å. The long-wave arms of such peaks extended above 2000 Å, increasing in amplitude with increase in size of the molecule. Because many spectrographs and spectrophotometers do not record below 2000–2200 Å, these long-wave limbs of bands of saturated compounds, rising in absorption with decreasing wave length, are often referred to as "end" absorption.

Compounds with single unsaturated bonds such as  $C=C$ ,  $C=O$ , or  $C=N$  were found to have absorption bands, usually weak, in the region 1900–3000 Å, the actual wave length being dependent on the adjacent parts of the molecule.

Strong absorption bands in the region 2000–4000 Å are always correlated with molecular structures containing chains or rings of conjugated double bonds; in general, the larger the conjugated structure, the stronger is the absorption and the longer the wave length of the maximum absorption. Ring structures with conjugated double bonds, as in aromatic compounds, often possess particularly high absorption.

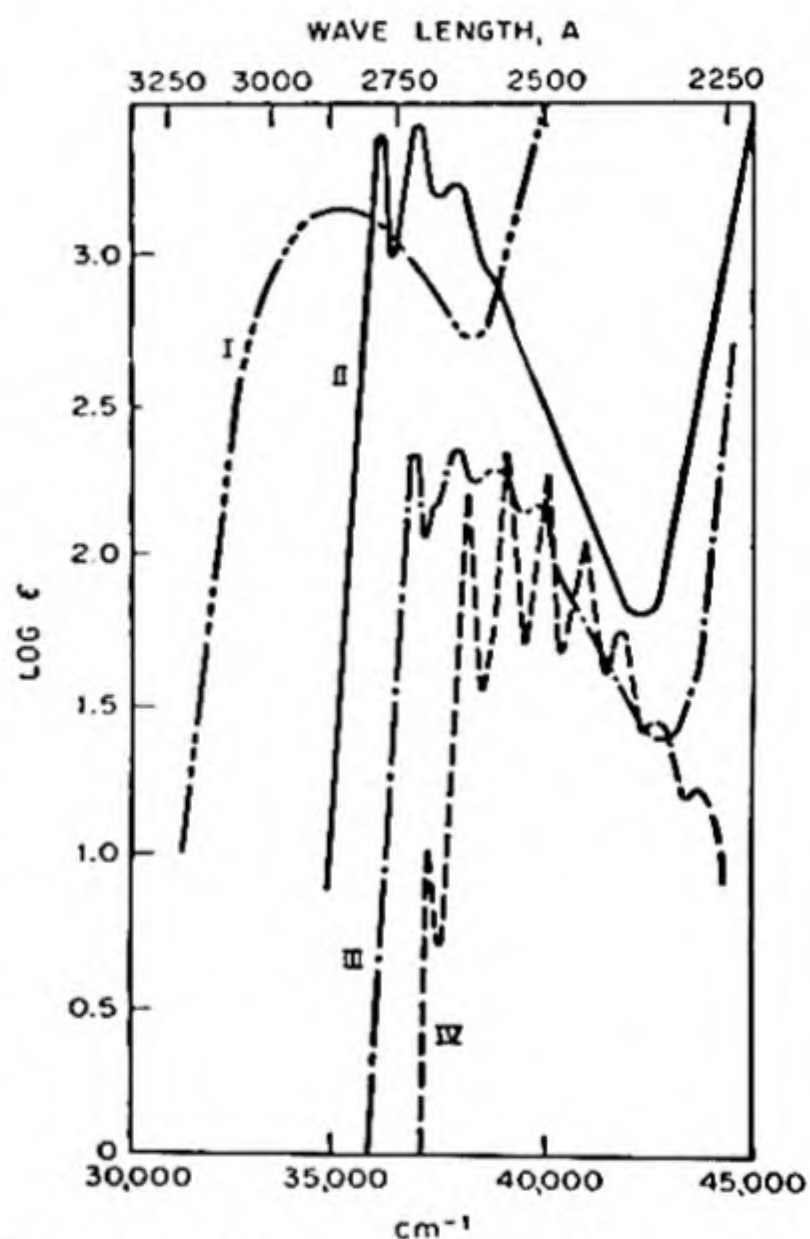


FIG. 5-4. Ultraviolet absorption spectra of some simple derivatives of benzene. I, aniline; II, phenol; III, chlorobenzene; IV, benzene; all in heptane. (*Adapted from Wolf and Herold, 1931.*)



The absorption of conjugated-bond groups separated within a given molecule by two or more saturated bonds is usually independent and simply additive. The absorption of such groups can, of course, be affected by the addition of side chains or auxiliary groups, especially if the latter may be charged ( $\text{NH}_2$  and  $\text{OH}$ ). Such groups may distinctly affect the spectral position and intensity of absorption of a given conjugated system (Fig. 5-4).

In large polar macromolecules, such as proteins and nucleic acids, the near-ultraviolet absorption spectrum of the polymer is often not strictly

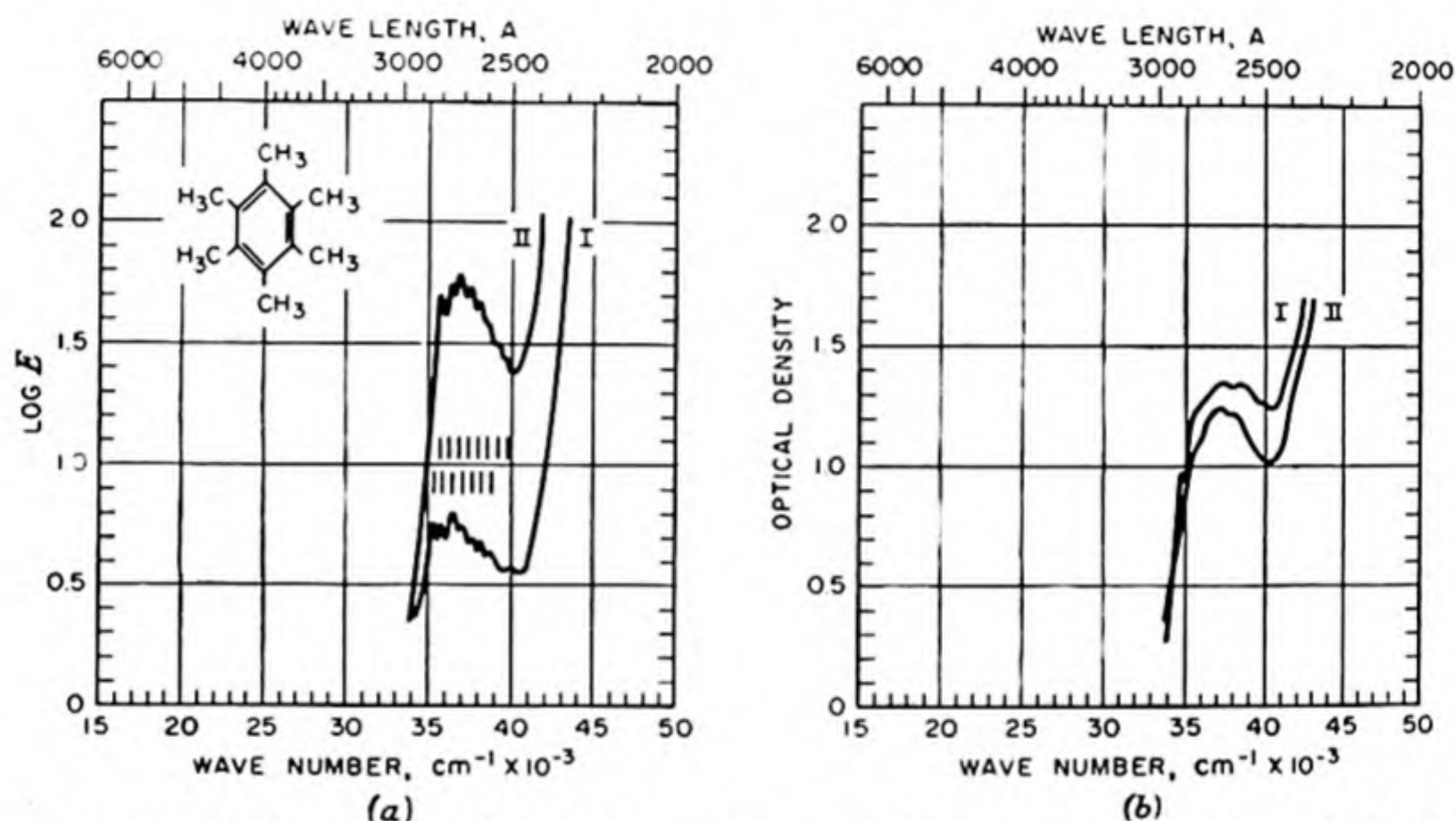


FIG. 5-5. (a) The ultraviolet dichroism of hexamethylbenzene crystals. I, Electric vector perpendicular to the plane of the ring; II, electric vector parallel to the plane of the ring. (b) The ultraviolet dichroism of tobacco mosaic virus particles, oriented by streaming. I, Electric vector perpendicular to the direction of streaming; II, electric vector parallel to the direction of streaming. (Scheibe *et al.*, 1943; see also Wilkins *et al.*, 1950.)

the linear sum of the absorption of its component conjugated groups, even though these component groups are separated by appreciable lengths of saturated bonds. This nonadditivity appears to be due to the formation of labile intergroup bonds to the conjugated groups, either of ionic or hydrogen-bond type. Thus the ultraviolet absorption spectra of proteins are often not simply the sum of the absorption of the component amino acids. The phenolic group of tyrosine, for instance, frequently appears to be involved in some type of loose bond (Crammer and Neuberger, 1943; Sizer and Peacock, 1947; Finkelstein and McLaren, 1949; Schauenstein and Treiber, 1950). The ultraviolet absorption of highly polymerized deoxyribonucleic acid is about 25–30 per cent less than that of the depolymerized form, which form is very nearly the linear sum of the absorption of the component nucleotides (Kunitz, 1950;

Tsuboi, 1950; Loofbourow, 1940; Sinsheimer, 1954). The absorption spectrum of ribonucleic acid also increases in intensity and shifts slightly toward shorter wave lengths during depolymerization (Kunitz, 1946; Tsuboi, 1950).

If the radiant energy is plane polarized, absorption will be greatest when the plane of the electric vector is parallel to the direction (*s*) of greatest electron mobility and maximal induced dipole moment, i.e., parallel to the direction of a chain of conjugated bonds, as in carotene, or parallel to the plane of the ring in a planar aromatic or heterocyclic molecule, as in benzene (Fig. 5-5). Spectrally distinct absorption bands may appear, corresponding to transitions involving mutually perpendicular changes in dipole moment (Lewis and Bigeleisen, 1943b; Scheibe and Kandler, 1938; Scheibe *et al.*, 1943; Coulson, 1948; Nakamoto, 1952). In solution the random orientation of molecules will prevent detection of any such preferred directions; however, in cellular structures or in crystals, uniform molecular orientation may permit a preferential absorption for light polarized in these directions (Butenandt *et al.*, 1942;

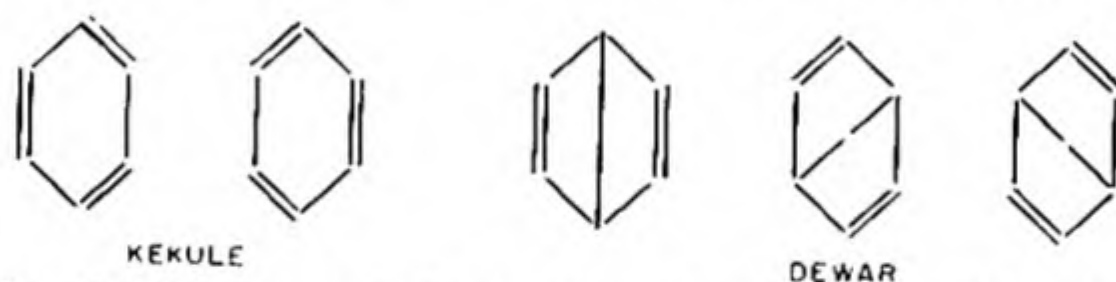


FIG. 5-6. The canonical structures of benzene. (Sklar, 1937.)

Schauenstein *et al.*, 1949). This phenomenon of differential absorption dependent on the plane of polarization is known as dichroism and can be useful as an indication of molecular orientation.

### THEORETICAL DEVELOPMENTS

As indicated, these empirical correlations have received support from modern theories of the electronic structure of organic molecules and of the change in electronic configuration attendant on the absorption of radiation. These treatments have developed along two lines, the valence-bond concept (Maccoll, 1947; Heitler, 1945; Pauling, 1945; Van Vleck and Sherman, 1935; Sklar, 1937) and the molecular-orbital concept (Coulson, 1947; Herzfeld, 1947; Mulliken and Rieke, 1941).

In the valence-bond concept, the electrons involved in chemical bonds are assumed to remain in atomic orbitals, which overlap with orbitals of the neighboring atoms, and the energy of each bond may be calculated from wave-mechanical principles. The energy level of the molecule is dependent on the summation of the energy levels of each bond. It is recognized that, with conjugated structures, the formulas usually written represent but one of several possible canonical forms (Fig. 5-6), all of which may be considered to contribute in varying degree to the actual



structure. By combining these possible forms in various proportions, a combination may be found which produces the lowest energy state. The energy level of this state is generally less than that of any of the individual canonical forms, a result known as "resonance stabilization" (Wheland, 1944) and due to "exchange energy" (Heitler, 1945). Other combinations, with varying proportions of the possible canonical forms, give rise to higher energy levels to which the molecule may be excited on the absorption of radiation.

Resonance among possible structures in the excited state can lower the energy of the excited state and thus reduce the energy difference between it and the ground state, thereby increasing the wave length of the absorption associated with the electronic transition. The intensity of absorption will be greatest for transitions between states involving resonating structures which have appreciable dipole moments.

The molecular-orbital method, which has been the more successful in regard to the correlation of calculated with observed spectra, has been based on the assumption of *molecular orbitals* for the valence electrons of the atoms involved in the chemical bonds. In this development the molecular orbitals are usually made up formally of linear combinations of appropriately chosen atomic orbitals—LCAO method<sup>1</sup> (Mulliken and Reike, 1941; Chirgwin and Coulson, 1950; Lennard-Jones, 1949; Matsen, 1950; Dewar, 1950; Platt, 1950; Longuet-Higgins *et al.*, 1950). The construction of the molecular orbitals may involve only the atomic orbitals of two atoms, as is usually the case with single bonds and with isolated double bonds, in which case they are referred to as "localized orbitals," or, as in the case of conjugated chains of double bonds, the molecular orbitals may involve contributions from the  $\pi$  orbitals of all the atoms involved in the chain. In this latter case, the orbital is said to be "unlocalized," and electrons in such orbitals are considered to migrate freely along the chain (Fig. 5-7).

Varying the combinations of atomic orbitals will produce molecular orbitals of various energy levels. The electrons available for bonding (all in the outer atomic shell) are then disposed in successively higher energy levels, two to a molecular orbital with spins opposed, until all electrons are accounted for. Absorption of radiation may then cause an excitation of an electron from the highest filled molecular orbital to the lowest unfilled orbital. The energy difference between orbital levels

<sup>1</sup> Refinements of this procedure involve the use of "antisymmetrized molecular orbitals" to reduce the apparent contribution of configurations including multiply ionized atoms (Goepfert-Mayer and Sklar, 1938; Roothaan, 1951) and recognition of configurational interaction (Jacobs, 1949; Craig, 1950). Another theoretical approach which has had considerable success is the "free-electron model" in which all the  $\pi$  electrons are considered to be able to migrate freely throughout the molecule, along the atomic bonds, in a potential field that is constant, or, in some instances, sinusoidally varying (Bayliss, 1948; Platt, 1949; Kuhn, 1949; Simpson, 1949).

decreases with increasing orbital energy. As a result, the transitions induced by absorption in large conjugated systems, which are transitions between high-level orbitals, require less energy, and thus a longer wave photon, than the transitions induced by absorption in smaller conjugated systems. This deduction accounts generally for the increase in wave length of the absorption peaks with increase in size of the conjugated system.

Absorption of radiation may also induce transitions from the highest filled molecular orbital to some of the higher unfilled orbitals; these tran-

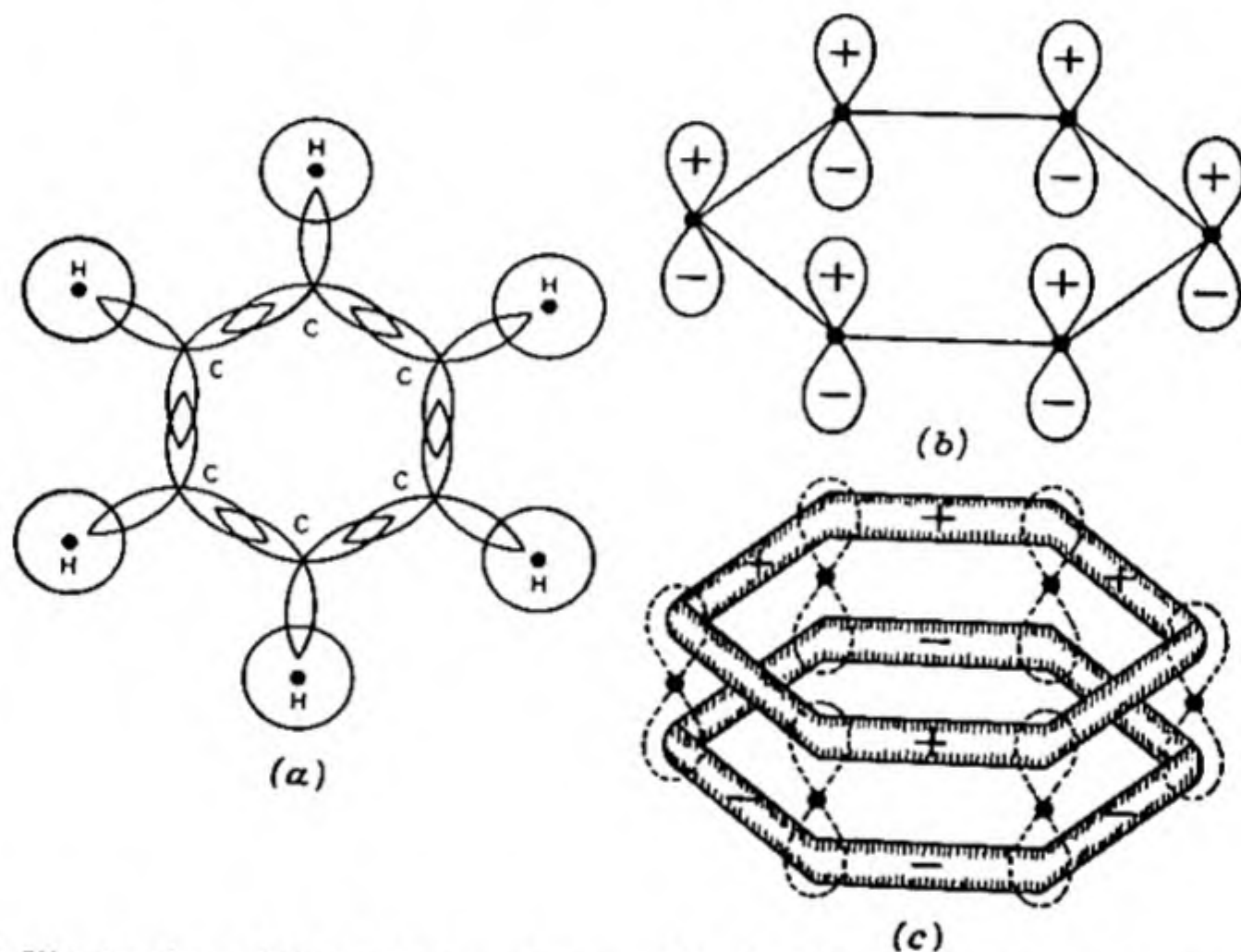


FIG. 5-7. Illustration of the  $\sigma$  and  $\pi$  atomic orbitals of benzene and the fusion of the  $\pi$  atomic orbitals to form the lowest energy molecular orbital. (a)  $\sigma$  orbitals; (b)  $\pi$  atomic orbitals; (c)  $\pi$  molecular orbitals. (Coulson, 1947.)

sitions correspond to absorption bands at shorter wave length than those of the bands just described.

The excitation induced by absorption usually involves the transition of an electron from a "bonding" to an "antibonding" orbital without change of spin direction.<sup>2</sup> The antibonding orbital introduces an additional nodal plane into the function specifying the probability distribution for the position of the electron. The absorption of double bonds or of conjugated chains involves excitation of a  $\pi$  electron, an electron of which the probability distribution already contains a node in the plane of the bond or chain. Hence excitation introduces a new node, which lies between the atoms of the bond or two of the atoms of the chain. Thus

<sup>2</sup> Transitions involving a change in the net electron spin of the molecule, i.e., "singlet-triplet transitions," are usually of low probability (Kasha, 1947).



for a double bond, in the excited state, there is a greater probability of the electron being on one or the other atom of the bond than of its being in the interatomic region. The probability distributions and associated

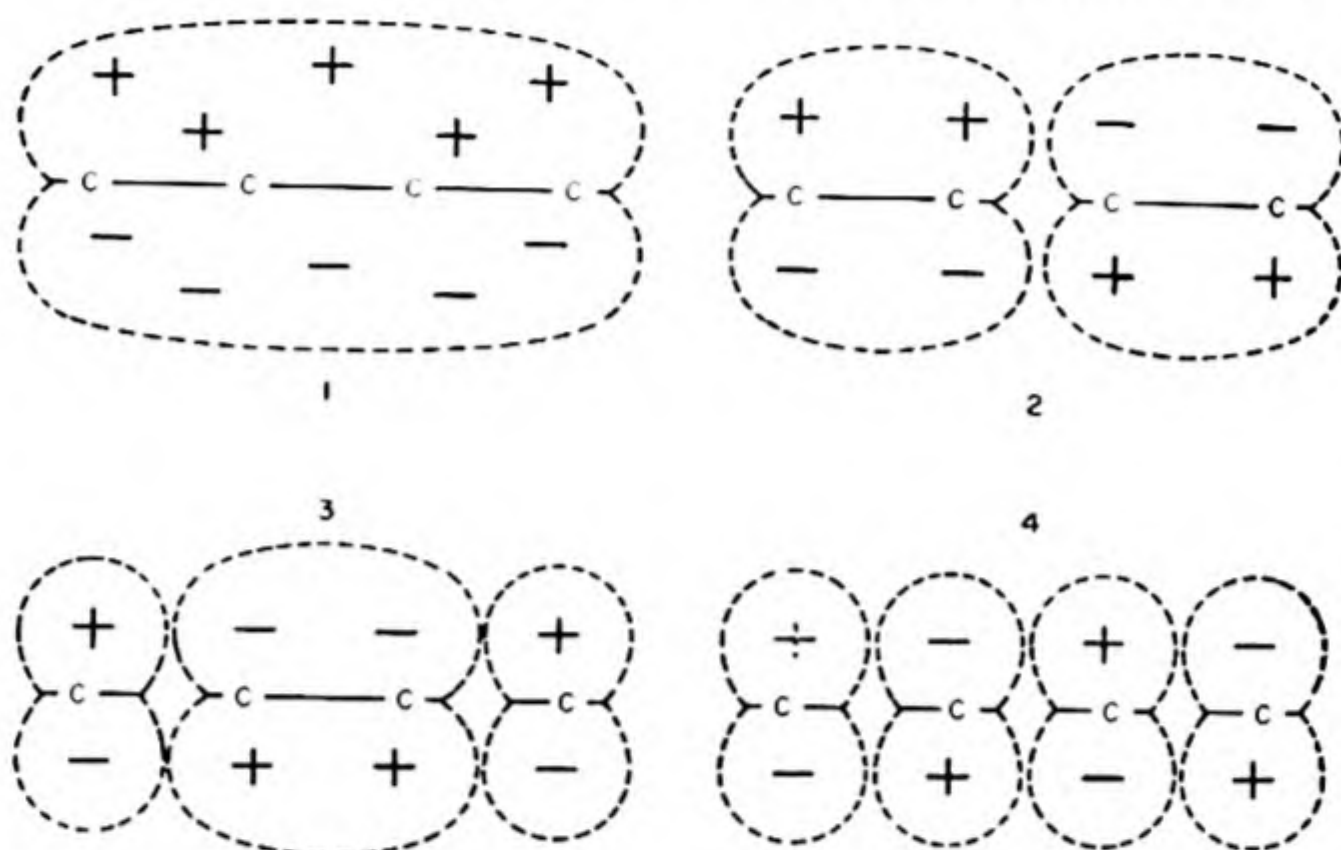


FIG. 5-8. A schematic representation of the wave functions describing the four molecular orbitals that may be synthesized by linear combinations of the four  $\pi$  atomic orbitals of butadiene  $\text{CH}_2=\text{CH}-\text{CH}=\text{CH}_2$ . On and outside the dotted lines the wave functions are practically zero. Within the dotted lines the wave functions have finite values, with sign as indicated, and thus these are the regions with an appreciable probability for the presence of an electron (the pluses and minuses refer only to the sign of the electronic wave function and not to the charge). (*Reproduced from Chemical Aspects of Light, by E. J. Bowen, copyright, 1946, by Oxford University Press.*)

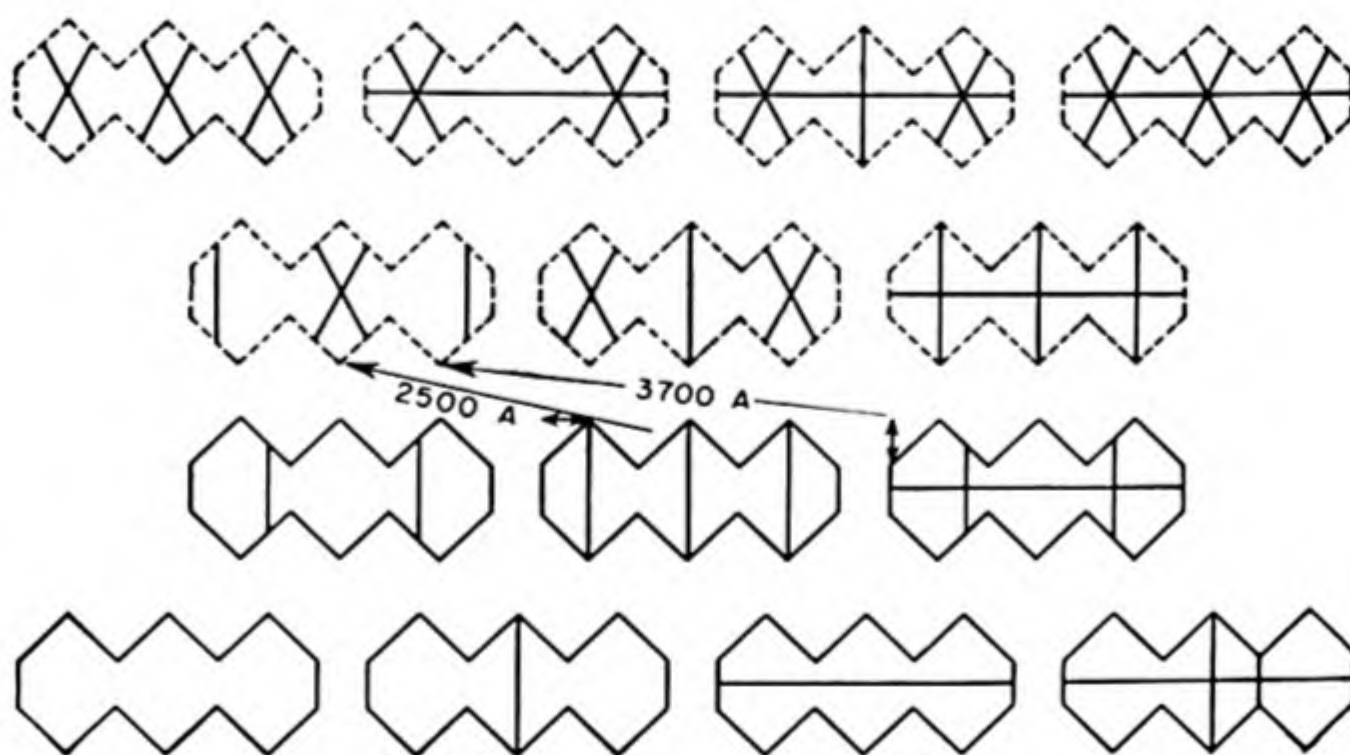


FIG. 5-9. The molecular orbitals of anthracene. (*Bowen, 1950.*)

nodal planes for the molecular orbitals of a simple conjugated chain (butadiene) are shown in Fig. 5-8 (Bowen, 1946). Absorption induces a transition of an electron from orbital 2 to orbital 3.

In aromatic molecules the new nodal plane may intersect the molecular

plane along either of two mutually perpendicular axes ( $x$ ,  $y$ ), producing different molecular orbitals. If the molecule is asymmetric (as in naphthalene), these orbitals will represent different energy levels, and transitions from the ground state to these orbitals will be associated with spectroscopically distinct absorption bands, which will be strongly dichroic (Coulson, 1948). The intersections of the nodal planes with the molecular plane for the molecular orbitals of anthracene are indicated in Fig. 5-9. The transitions that correspond to the two prominent ultraviolet absorption bands of anthracene are labeled with the band wave length.

It can be shown that these two approaches, valence-bond and molecular-orbital, in their simple forms, probably bracket the correct solution; the valence-bond method does not allow enough weight to possible ionic structures, i.e., those in which two or more of the bonding electrons may be concentrated on one atom; the molecular-orbital method allows too much. More advanced developments of both theories have tended to narrow the gap between them.

### SPECIFICATION OF ABSORPTION INTENSITY

The ultraviolet absorption spectra of substances of biochemical interest are usually obtained with solutions of these substances in transparent solvents. The measured absorption at any wave length will then be dependent on the concentration of the substance in the solution and the length of the light path in the solution. The specification of absorption spectra may be standardized by referring all measured spectra to the spectrum that would be obtained from a solution of a standard concentration and a standard light path. This conversion of measured to standard spectrum is rendered easy by the simple nature of the formula relating absorption to concentration and light path.

Since the absorption of a photon by a molecule is an all-or-none act and since all molecules may be assumed to have, statistically, the same probability of absorption of an incident photon of a given wave length, any layer of a solution of thickness  $dl$ , transverse to the light beam, may be expected to absorb the same fraction of radiant energy of one wave length as any other such layer, and if  $dl$  is small, this fraction will be proportional to  $dl$ . Thus

$$-\frac{dI}{I} \propto dl.$$

This statement is known variously as Lambert's or Bouguer's law.

If the absorbing molecules may be assumed to act independently, the fraction of incident energy absorbed in a given layer will be expected to be proportional to the concentration of absorbing molecules in the solution.



Combining this with the previous expectation,

$$-\frac{dI}{I} \propto cl$$

is obtained. This relation is known variously as the Lambert-Beer or Bouguer-Beer law.

Integrating, it is found that

$$\int_{I_0}^I -\frac{dI}{I} \propto \int_0^l c \, dl,$$

$$\ln \frac{I_0}{I} \propto cl,$$

or also

$$\log_{10} \frac{I_0}{I} \propto cl.$$

The proportionality constant in this equation, which applies at each wave length, is, of course, characteristic of the absorbing substance. Numerically, it will depend on the logarithmic base employed and on the

TABLE 5-1. SYMBOLS FOR THE PROPORTIONALITY CONSTANT

Log base	Optical path length ( <i>l</i> ), cm		
	<i>c</i> , g/liter	<i>c</i> , moles/liter	<i>c</i> , moles/ml
<i>e</i>	$\mu$	<i>E</i>	$\beta$
10	<i>k</i> ; <i>a<sub>s</sub></i> (specific extinction)	$\epsilon$ ; <i>a<sub>m</sub></i> (molar extinction)	

units for concentration (*c*) and optical path length (*l*). Various combinations of base and units have been employed, and the most commonly used symbols for the proportionality constant in the various systems are indicated in Table 5-1. Thus

$$\epsilon = \frac{\log_{10} (I_0/I)}{cl}$$

where *c* is in moles per liter and *l* is in centimeters.

The quantity  $\log_{10} (I_0/I) = \epsilon cl$  is often referred to as the optical density<sup>3</sup> (*D*) of the solution. Thus  $\epsilon$  is the optical density that would be measured for a 1-cm path of a solution containing 1 mole/liter.<sup>4,5</sup>

<sup>3</sup> Also referred to as the extinction or the absorbance (Gibson, 1949; Brode, 1949).

<sup>4</sup> Another symbol occasionally used to specify absorption is  $E_l^p$  which signifies the optical density of an *l*-cm path of a solution containing *p* per cent of the absorbing substance.

<sup>5</sup> In some circumstances,  $\log_{10} \epsilon$  or  $\log_{10} D$  may be plotted either to compress a wide range of values into reasonable dimensions or to obtain a curve the shape of which is independent of the concentration or path length, which may be unknown. Since  $\log_{10} D = \log_{10} \epsilon + \log_{10} c + \log_{10} l$ , the latter will enter only as additive constants.

The Lambert or Bouguer law is valid under all conditions of normal use. The modification due to Beer is accurate as long as the condition for its validity is met—that the absorbing molecules act independently. In concentrated solutions there is often a tendency toward dimerization or other forms of molecular association. In these instances the nature of the absorbing entity really changes with changing concentration, so that Beer's law will not apply.

Another method of indication of the absorbing power of a substance is the specification of the "absorption cross section" ( $\sigma$ ) of molecules of the substance as a function of wave length. The absorption cross section is a measure of the probability of absorption of a photon, known to be crossing a unit area transverse to the beam, by a single molecule known to be confined within that area. This quantity is useful in calculations concerning the possibility of radiative-energy transfer from the primary receptor to other receptors within a cell (Arnold and Oppenheimer, 1950).

The absorption cross section is related to  $\epsilon$  by the following formula:

$$\sigma = 3.83 \times 10^{-21} \epsilon$$

where  $\sigma$  is in square centimeters. The cross section  $\sigma$  does not necessarily bear simple relation to the physical cross-sectional area of the molecule, although in certain instances a good correlation has been demonstrated between an "effective" geometrical cross section and absorptive power (Braude, 1950).

### SPECTRAL POSITION

The position of an absorption band in the electromagnetic spectrum is usually defined by the position of the absorption maximum. This position may be expressed in terms of (1) wave length, in Angstroms ( $1 \text{ \AA} = 10^{-8} \text{ cm}$ ) or millimicrons ( $1 \text{ m}\mu = 10^{-7} \text{ cm}$ ); (2) wave number,<sup>6</sup> in  $\text{cm}^{-1}$  or  $\text{mm}^{-1}$ ; or (3) frequency, in vibrations per second or in fresnels ( $1 \text{ f} = 10^{12} \text{ vps}$ ).

Thus the longer wave maximum in the absorption spectrum of methylcholanthrene at 77°K occurs at 2995  $\text{\AA}$ , 299.5  $\text{m}\mu$ , 33,390  $\text{cm}^{-1}$ ,  $10.02 \times 10^{15} \text{ vps}$ , or 10,020 f.

### BAND WIDTH

The width of an absorption band is usually considered to be the spectral separation between the points of half-maximal absorption. This separation may be expressed in any of the units used to express the position of the absorption maximum.

<sup>6</sup> The wave number is defined as the number of wave lengths per centimeter (or millimeter) of path *in vacuo*.



## ENVIRONMENTAL FACTORS INFLUENCING ABSORPTION

In general, a variation of any factor that influences the electronic configuration of the absorbing molecules, either uniformly or with a statistical distribution, will affect the absorption spectrum (Sheppard, 1942). In addition, certain factors may alter the technical conditions of the absorption measurement and thereby affect the spectrum.

## SOLVENT

The choice of solvent can influence the position, width, and intensity of absorption bands. Changes in position are to some degree correlated

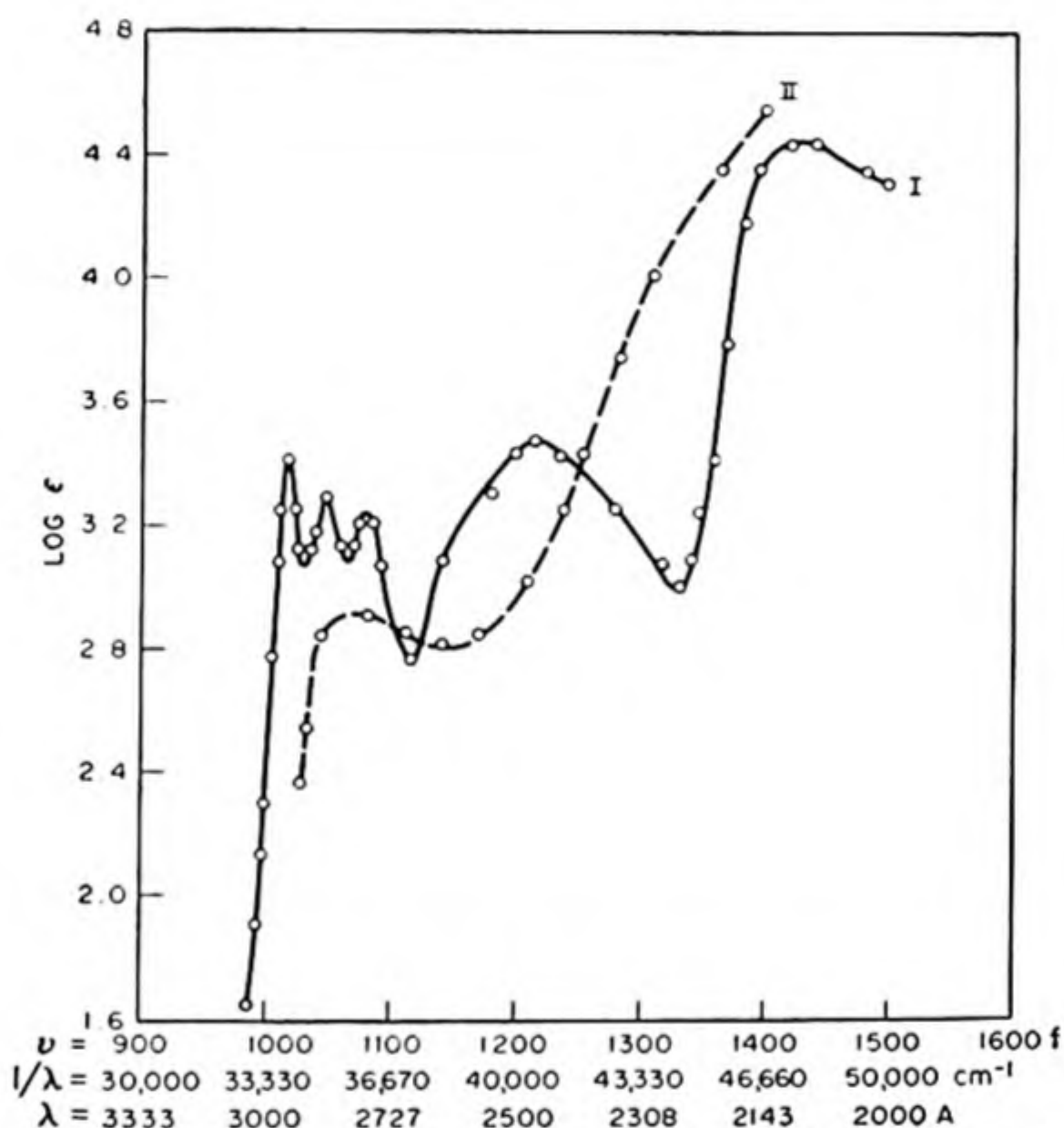


FIG. 5-10. The absorption spectrum of phthalic anhydride. I, in hexane; II, in alcohol. (Menczel, 1927.)

with the dielectric constant of the solvent according to Kundt's rule which states that, with increasing dispersion of the solvent, the absorption maximum is shifted toward longer wave lengths. Although Kundt's rule is generally valid for nonpolar solvents, there are serious deviations with polar solvents (Sheppard, 1942).

This shift may be interpreted as indicative of the increased role played by ionized structures in resonance stabilization of the excited state in media of high dielectric constant (Wheland, 1944) or in terms of the influence of the reaction field of the oscillating dipole on the electric field

of the light wave in a dielectric medium (Bayliss, 1950; Hartmann and Schlafer, 1950).

As indicated, absorption bands are widened in polar solvents because of increased molecular interaction with consequent perturbation of the electronic configurations (Fig. 5-10).

The total absorption intensity, the  $\int \epsilon d\nu$ , can also vary with the solvent and would be expected, in general, to increase moderately with increasing solvent refractive index (Chako, 1934) owing to augmentation of the

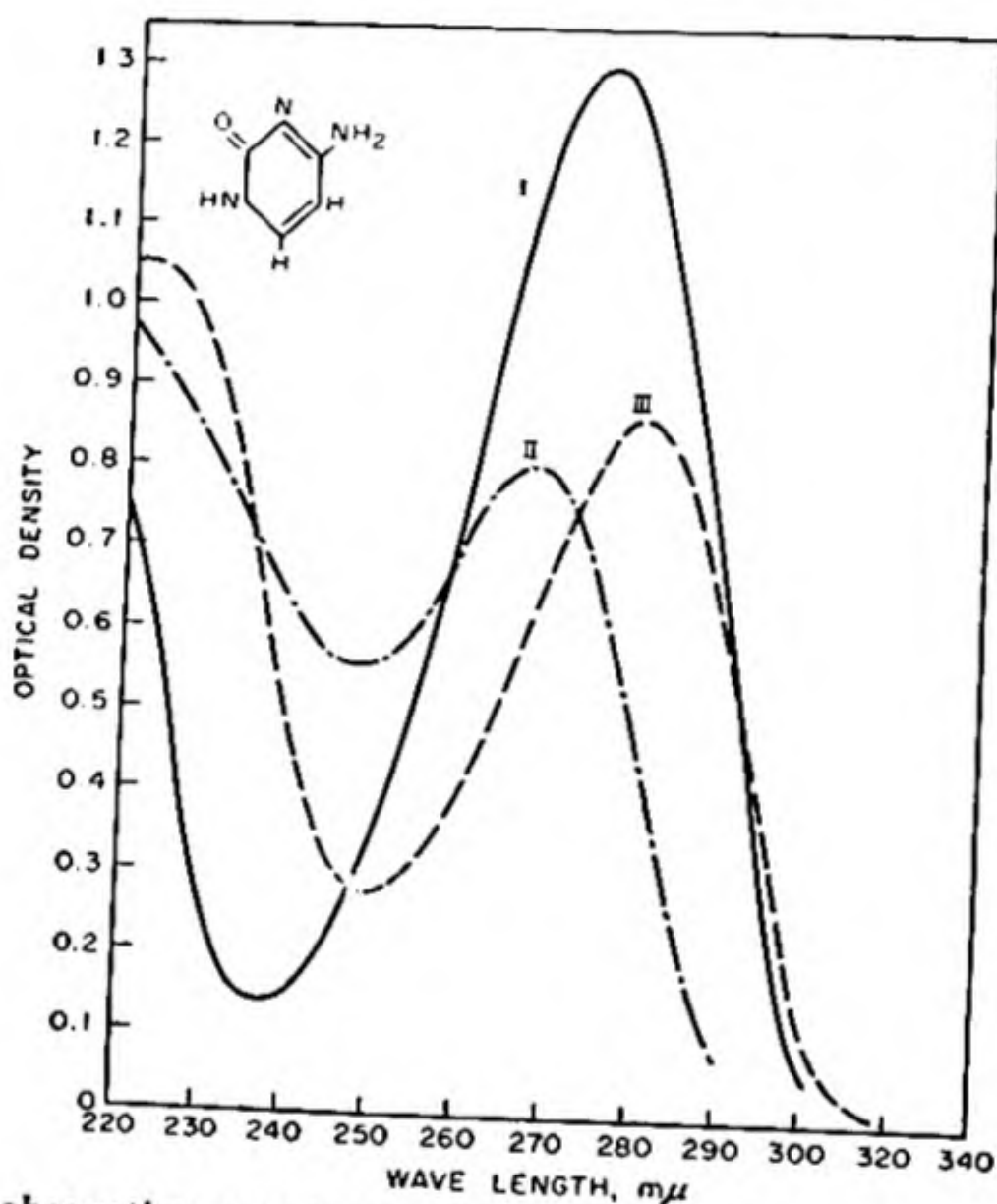


FIG. 5-11. The absorption spectrum of cytosine at three different values of pH ( $\epsilon = 8.06 \times 10^3 D$ ). I, pH 1.2; II, pH 6.0; III, pH 12.7. (Scott, unpublished data, 1951.)

exciting electric field by the field of the induced dipoles in the medium. However, this expectation is frequently not fulfilled for unknown reasons (Jacobs and Platt, 1948).

#### pH VALUE

In aqueous solutions of substances containing dissociable groups, the pH of the solution will usually have a marked effect on the absorption spectrum. Ionization of any such group, resulting in gain or loss of charge, will certainly alter the basic electronic configuration of the molecule and thereby the spectral distribution of absorption. An example is the absorption of a solution of cytosine at various pH values (Fig. 5-11),



indicating the effects of ionization of the amino group ( $pK = 4.60$ ) and of the enolic group ( $pK = 12.16$ ) (Levene and Bass, 1931).

### CONCENTRATION

As was mentioned in the discussion of Beer's law, in concentrated solutions the association of solute molecules may cause modification of their absorption spectrum. This effect may give rise to a nonlinear relation between the optical density of such solutions at certain wave lengths and

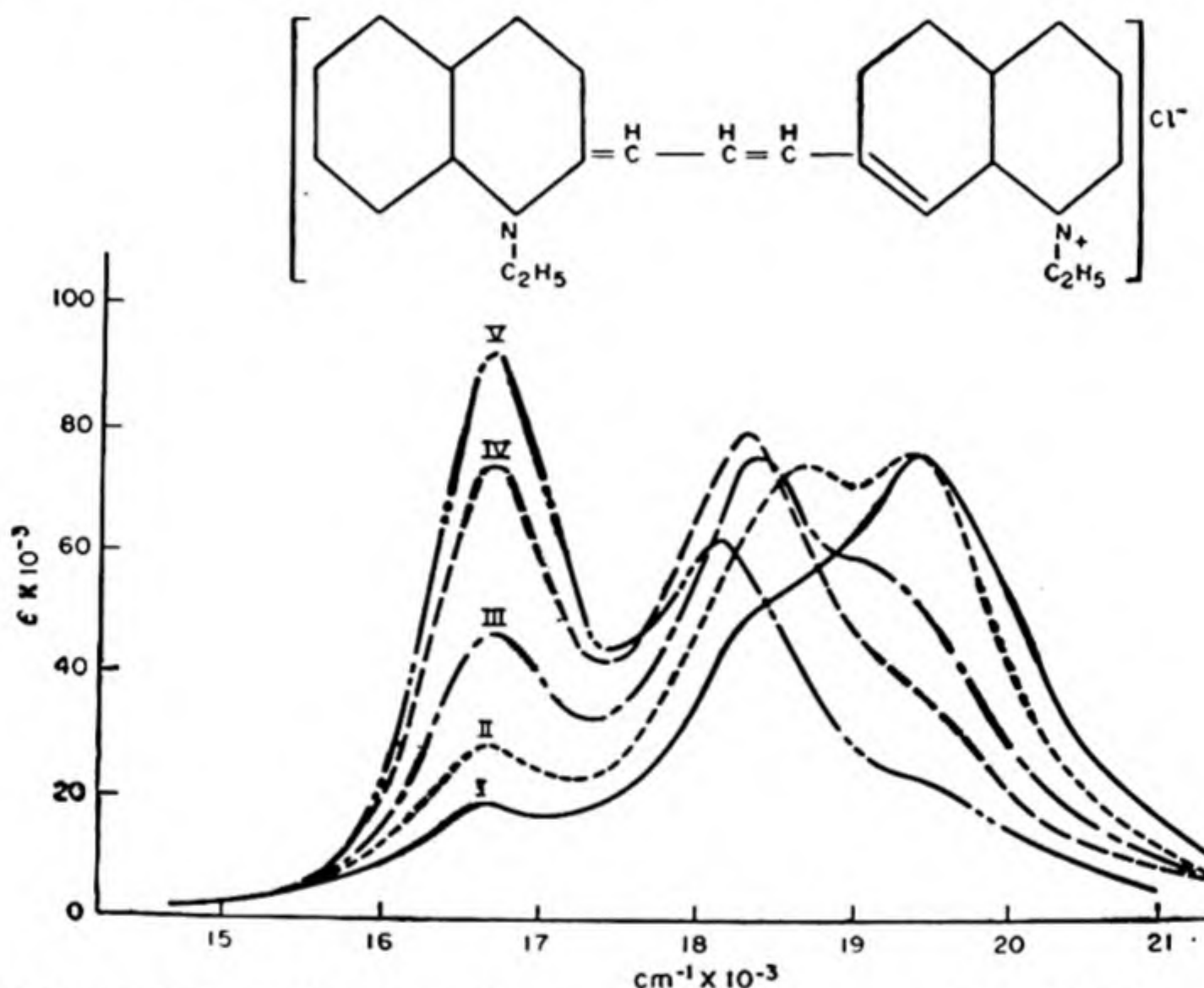


FIG. 5-12. Variation of the absorption spectrum of pinacyanol chlorides in water at  $20.0^\circ C$  with concentration. I,  $4.44 \times 10^{-4} M$ ; II,  $1.33 \times 10^{-4} M$ ; III,  $4.44 \times 10^{-5} M$ ; IV,  $1.33 \times 10^{-5} M$ ; V,  $4.44 \times 10^{-6} M$ . (Scheibe, 1938; reproduced from *Kolloid-Zeitschrift*.)

the solute concentration, as is observed with the Nessler test for ammonia (Hawk *et al.*, 1947).

In more extreme cases, extensive molecular association, possibly involving electron transfer through intermolecular hydration, may cause the development of entirely new absorption bands. An example of this is the "mesophase" J band (Fig. 5-12) of the cyanine dyes (Sheppard, 1942).

### TEMPERATURE

The temperature of an absorbing substance significantly affects its absorption spectrum by controlling the statistical distribution of molecules among various vibrational energy states associated with the lower energy electronic state and by influencing the velocity of Brownian

motion, which in turn determines the frequency of molecular collision. The latter influences the duration of the excited state and the extent of the distortion of the molecular electronic configurations by the electromagnetic fields of neighboring molecules. In addition, variation of temperature may vary the relative statistical contribution of various possible tautomers (Freed and Sancier, 1951) or resonating states. In general, reduction of the temperature, by reduction of the variety of initial energy levels in an electronic transition induced by absorption and by reduction of the perturbing effect of extramolecular fields, will reduce the width of the individual and fused absorption bands (Fig. 5-13). This effect may be particularly marked if the absorbing substance is in a crystalline

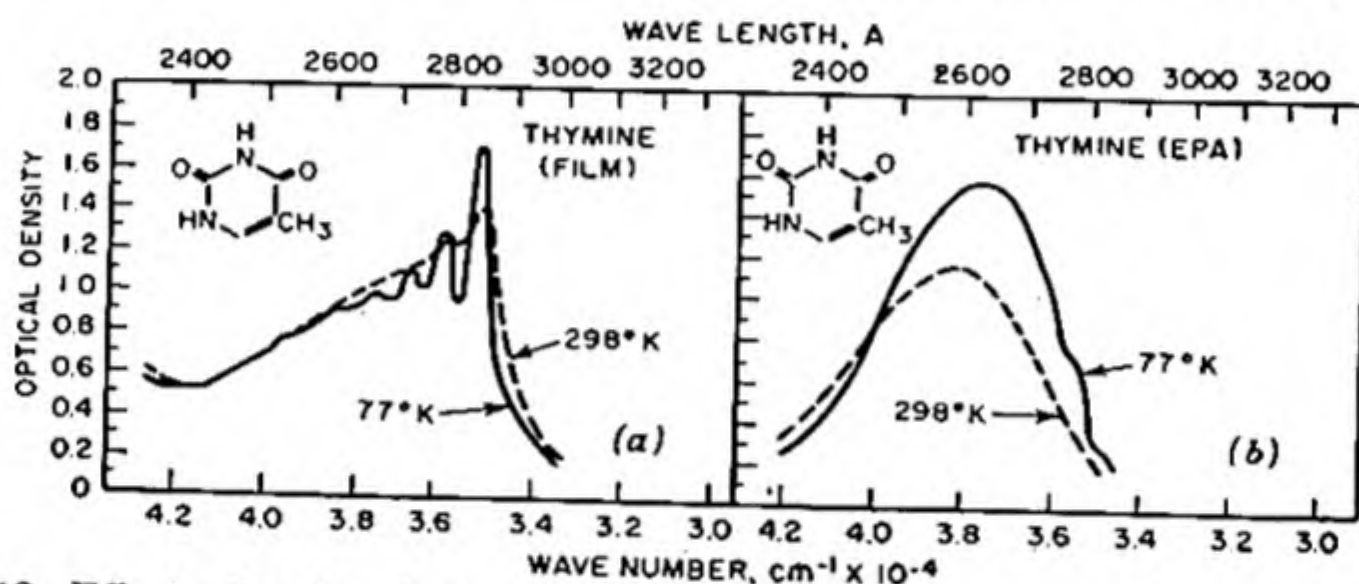


FIG. 5-13. Effect of reduced temperature on the absorption spectrum of thymine. (Sinsheimer *et al.*, 1950b.)

form so that the molecules have a uniform environment (Scott *et al.*, 1952).

### ORIENTATION

If the molecules of a dichroic substance are uniformly orientated, as might occur in a cellular structure, the absorption spectrum would depend on the plane of polarization of the incident radiation. If unpolarized light is used, as is ordinarily the case, the influence of the molecular orientation on the observed spectrum will depend on the degree of dichroism. In an extreme case, such as might occur if there were no absorption at all of light for which the electric vector lay in a particular plane, the maximum possible light absorption would be 50 per cent, corresponding to an optical density of 0.3 at that wave length (Commoner and Lipkin, 1949). Obviously, intermediate cases would permit various maximal values of optical density.

### SCATTERING

If the absorbing solution (or living cell) contains objects of dimensions comparable with those of the wave length of light employed, appreciable quantities of light may be lost from the beam by scattering out, as well as



by absorption. For simple spherical particles of diameter  $< \lambda/10$  such scattering may be expected to vary as  $1/\lambda^4$ . For larger and for more irregularly shaped particles, the variation of scattering power with angle and with wave length depend intimately on the particle size and shape; this is, in fact, the basis of molecular size and shape determination by means of light scattering (Oster, 1948; Doty and Steiner, 1950).

In any event, scattering will tend to obscure seriously the true absorption spectrum (Schramm and Dannenburg, 1944). If it is necessary to determine the absorption of turbid media or coarse structures, the effects of scattering may be minimized by use of a detector designed to capture as much scattered light as possible (Caspersson, 1950) and by use of a fluid medium with a refractive index as closely matched to that of the scattering substance as possible (Mitchell, 1950). In some instances it is possible to introduce a reasonable correction factor by extrapolation of data from wave length outside the absorption band (Treiber and Schauenstein, 1949).

#### LOCAL CONCENTRATIONS

In general, solutions will have a uniform distribution of the absorbing substance, but this situation is not necessarily true of cellular structures. If the substance tends to molecular association and deviation from Beer's law, the presence of local concentrations may appreciably alter the absorption spectrum. In addition to this potential effect, the aggregation of the absorbing molecules into discrete groups (possibly submicroscopic) will influence the absorption by virtue of the nonabsorbing "holes" left between the absorbing groups. The effect on the spectrum in this instance is similar to that described in the contingency of marked dichroism. If there is an appreciable chance of a photon passing through the specimen without encountering one of the postulated absorbing centers, then there will be a maximum possible absorption, independent of the amount of absorbing substance present.

#### ULTRAVIOLET ABSORPTION SPECTRA OF IMPORTANT BIOLOGICAL SUBSTANCES

Since the principal role of absorption spectra in radiation biology is to serve, within the limits suggested in the preceding section, as a key to the interpretation of the action spectra for various photobiological effects, it is useful to have a summary of the absorption characteristics of the principal known ultraviolet chromophores in living systems (Brode, 1946; Morton, 1942; Loofbourow, 1940, 1943; Ellinger, 1937, 1938; Miller, 1939).

##### PROTEINS AND AMINO ACIDS

It is convenient to consider separately the ultraviolet absorption of proteins in the region below 2400 Å and in the region above—generally

2400–3000 Å. All proteins show absorption below 2400 Å, increasing rapidly toward shorter wave lengths. Although certain amino acids, histidine, and tryptophane (Coulter *et al.*, 1936) and tyrosine (Smith, 1928) show characteristic peaks in this region, these peaks are generally submerged by the fast rising “end” absorption; this absorption is due in large part to the peptide bond (Magill *et al.*, 1937; Setlow and Guild, 1951; Goldfarb *et al.*, 1951) and to a lesser degree to absorption representing the long wave limb of the vacuum ultraviolet absorption peaks of single C—C, C—N, etc., bonds (Marenzi and Vilallonga, 1941a). The 223-m $\mu$  peak of tyrosine, which shifts to 242 m $\mu$  in alkaline medium (Kretchmer and Taylor, 1950), should then become apparent, but it does not always become apparent, probably because of binding of the phenolic group within the protein (Sizer and Peacock, 1947; Finkelstein and McLaren, 1949).

Absorption in the region 2400–3000 Å is due to the presence of the aromatic amino acids, phenylalanine, tyrosine, and tryptophane, especially the last two (Figs. 5-14, 15) (Goodwin and Morton, 1946). Proteins and polypeptides that lack these amino acids, such as gelatin (Loofbourow *et al.*, 1949) and clupein (McLaren, 1949), have negligible absorption in this region, although the peptide bond does have a very weak band at about 2800 Å ( $\epsilon = 1-5$ ) (Setlow and Guild, 1951).

Iodination of tyrosine, as in diiodotyrosine, induces a shift of the alkaline tyrosine band to 3115 Å (Marenzi and Vilallonga, 1941b); the alkaline absorption maximum of thyroxine is still further displaced to 3310 Å (Heidt, 1936; Marenzi and Vilallonga, 1941b, c).

### NUCLEIC ACIDS AND NUCLEOTIDES

The ultraviolet absorption spectrum of nucleic acids and polynucleotides is characterized by a strong absorption maximum at about 2600 Å, a minimum at about 2300 Å, and continuously rising “end” absorption at below 2300 Å. (Loofbourow, 1940; Hotchkiss, 1948; Ploeser and Loring, 1949; Schlenk, 1949). As has been noted, the spectra of the highly polymerized nucleic acids are not the linear sums of the spectra of their

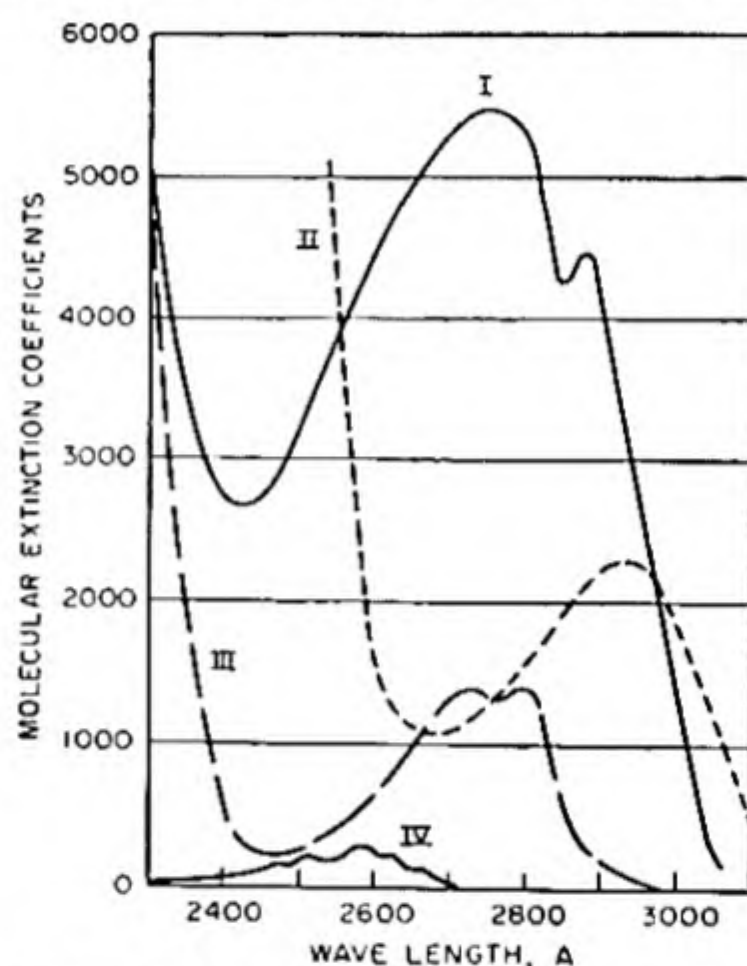


FIG. 5-14. Ultraviolet absorption spectra of the aromatic amino acids. I, tryptophane in *N*/10 HCl; II, tyrosine in *N*/10 NaOH; III, tyrosine in *N*/10 HCl; IV, DL- $\beta$ -phenylalanine. (Loofbourow, 1940.)



component nucleotides but are appreciably less, suggesting weak inter-nucleotide linkages, affecting the absorbing structures (Fig. 5-16).

The spectra of the individual nucleotides are easily distinguished (Fig. 5-17); they are generally similar to the spectra of the component purine and pyrimidine bases, which bases are primarily responsible for the absorption of these compounds in the spectral region above 2300 Å. The

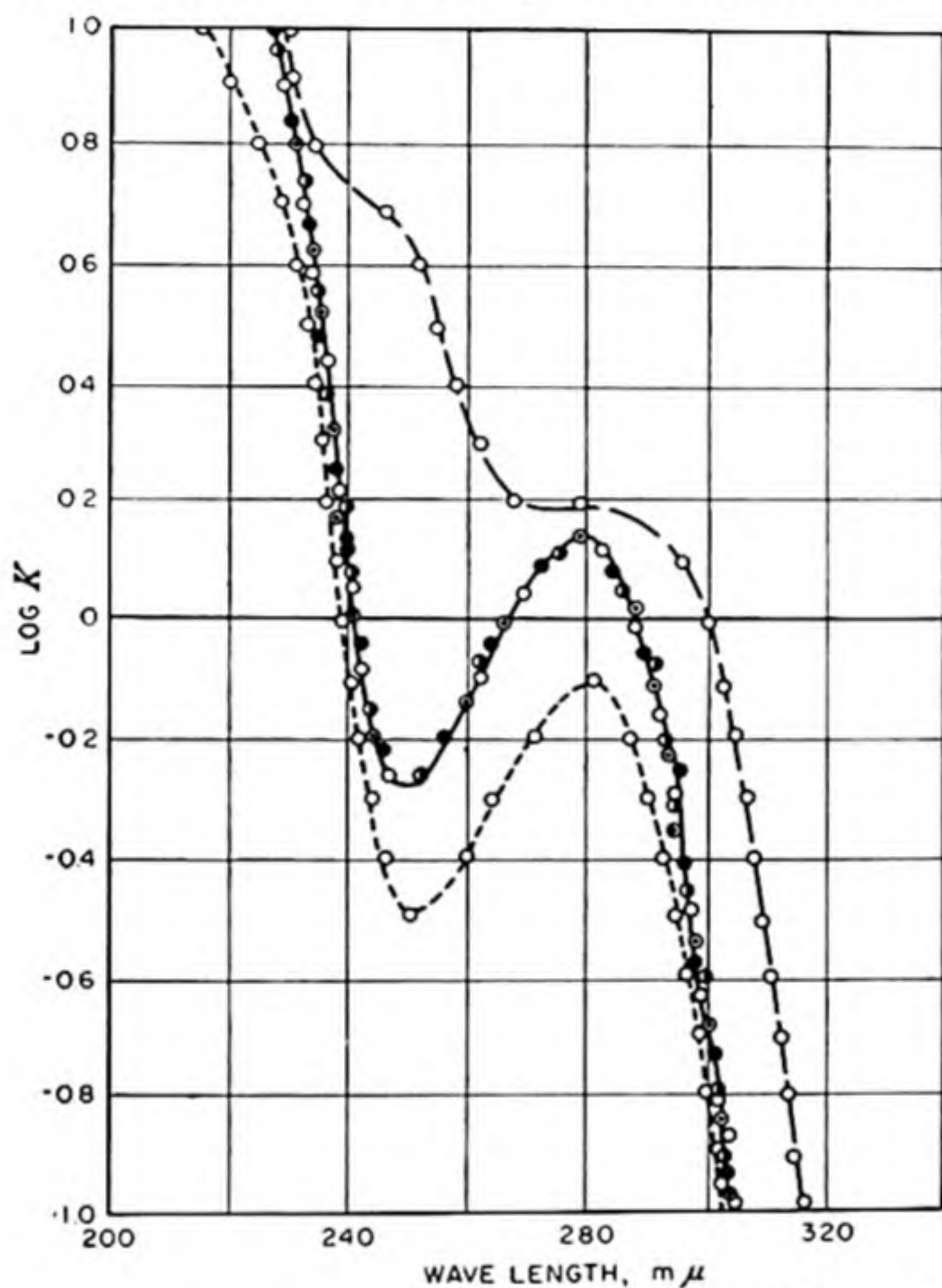


FIG. 5-15. Absorption spectrum of trypsin in acid and in alkaline solution, and of the trypsin-trypsin inhibitor complex ( $\epsilon = 36,700$  K). ●—●, trypsin in  $N/100$   $H_2SO_4$ ; ○—○, trypsin in  $N/10$   $HCl$ ; ⊙—⊙, trypsin after 24 hours in  $N/10$   $HCl$ ; ●—●, heat-inactivated trypsin; ○—○, trypsin in  $N/10$   $NaOH$ ; ○---○, trypsin inhibitor complex. (Schormüller, 1949.)

spectra are sensitive to changes in pH, especially in the regions of the pK values of the functional groups attached to the purines and pyrimidines (Stimson, 1949). Absorption in these bases is considered to be dependent on the presence of  $-C=C-C=N-$  or  $-C=C-C=O$  groupings (Cavalieri and Bendich, 1950).

### STERIODS

Although all steroids will exhibit end absorption in the region below 2100 Å, only those steroids that contain sequences of conjugated double

bonds will show appreciable absorption in the region 2200–3000 Å (Dannenberg, 1939; Fieser and Fieser, 1949). Thus steroids that contain only isolated ketone groups (androsterone) have only a very weak absorp-

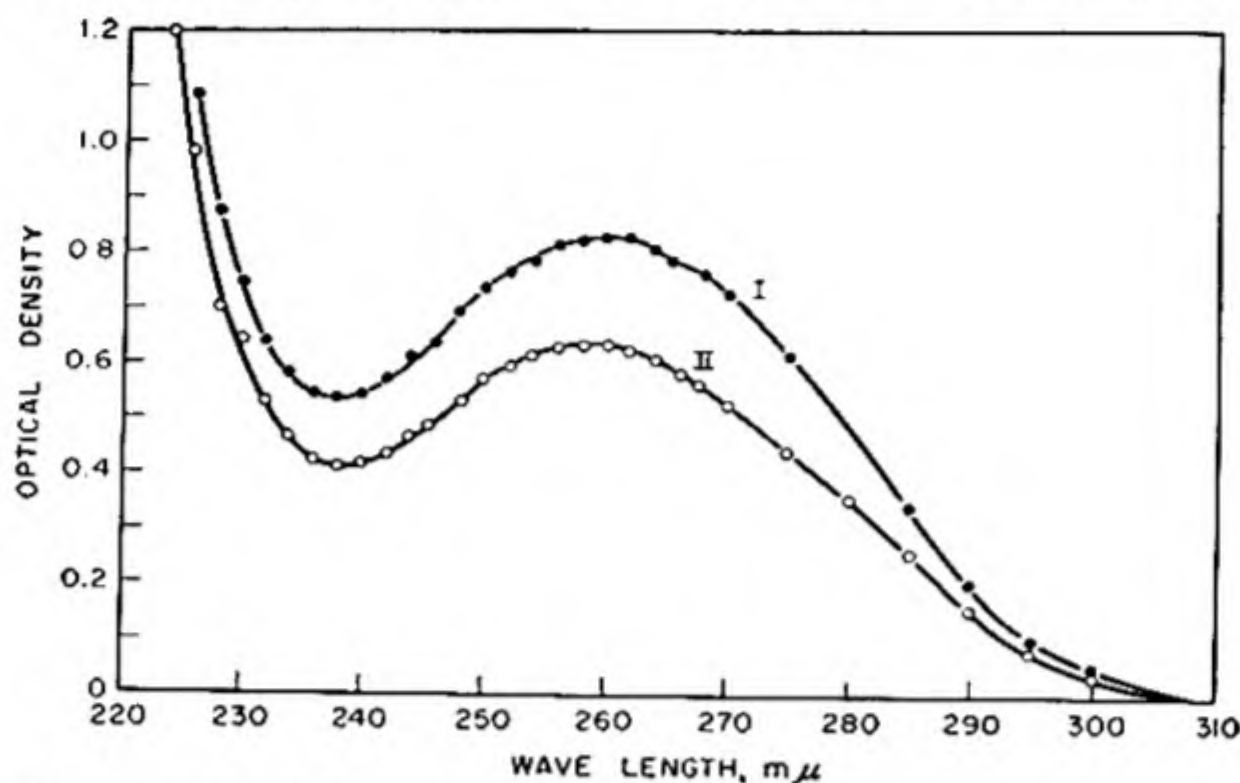


FIG. 5-16. Change in ultraviolet absorption spectrum of thymus deoxyribonucleic acid upon digestion with deoxyribonuclease. I, digested; II, undigested. (Kunitz, 1950, reproduced from the *Journal of General Physiology*.)

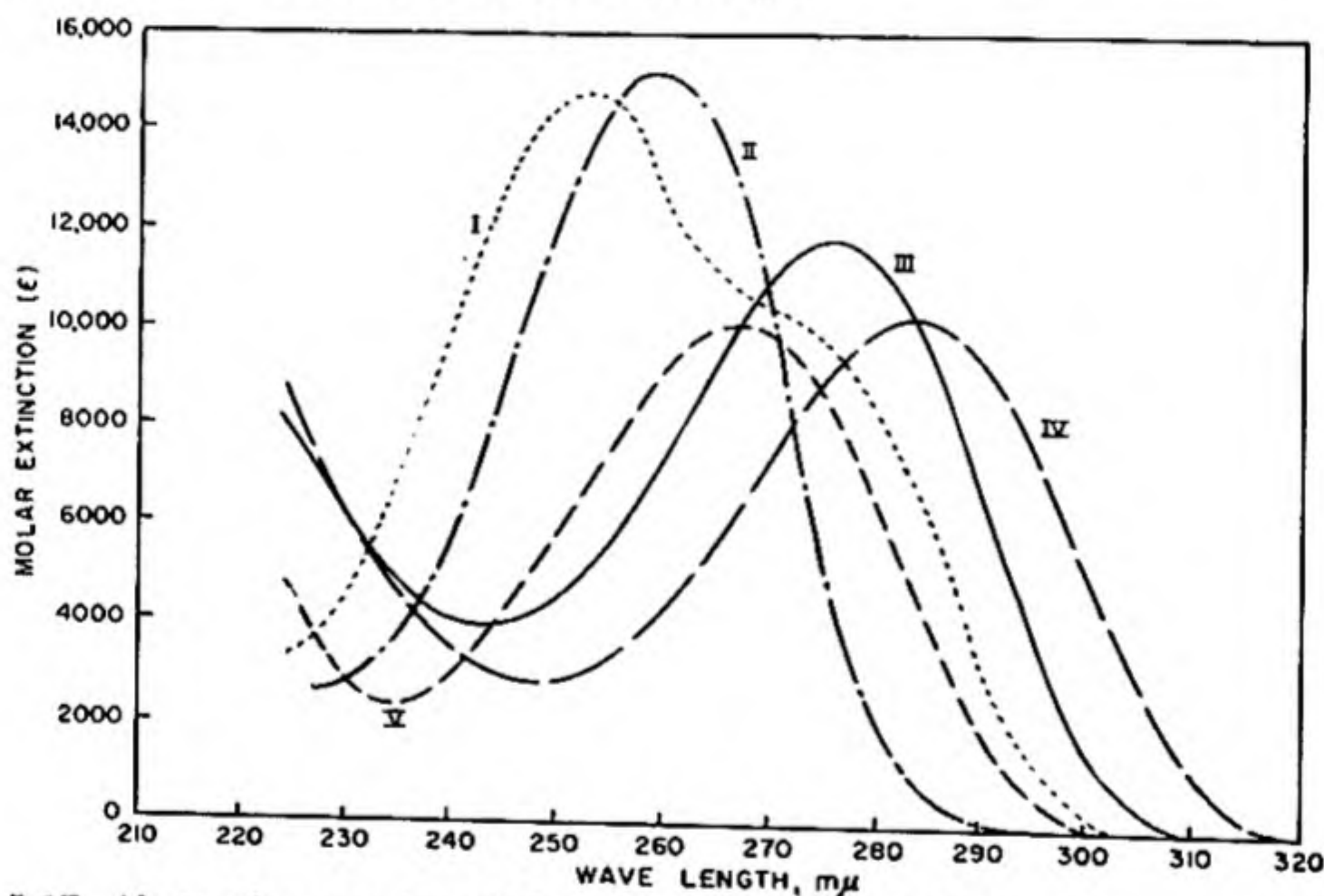


FIG. 5-17. Absorption spectra of five deoxyribonucleotides at pH 4.30. I, deoxyguanylic acid; II, deoxyadenylic acid; III, deoxycytidylic acid; IV, deoxy-5-methylcytidylic acid; V, thymidylic acid. (Sinsheimer, 1954.)

tion at about 2900 Å ( $\epsilon = 43$ ). Steroids with  $\alpha$  and  $\beta$  unsaturated ketones will have a strong absorption in the region 2300–2600 Å (for progesterone,  $\lambda_{\text{max.}} = 2370$  Å, and  $\epsilon = 17,000$ ) and a much weaker band near 3200 Å ( $\epsilon \approx 10\text{--}20$ ) (Morton, 1942).



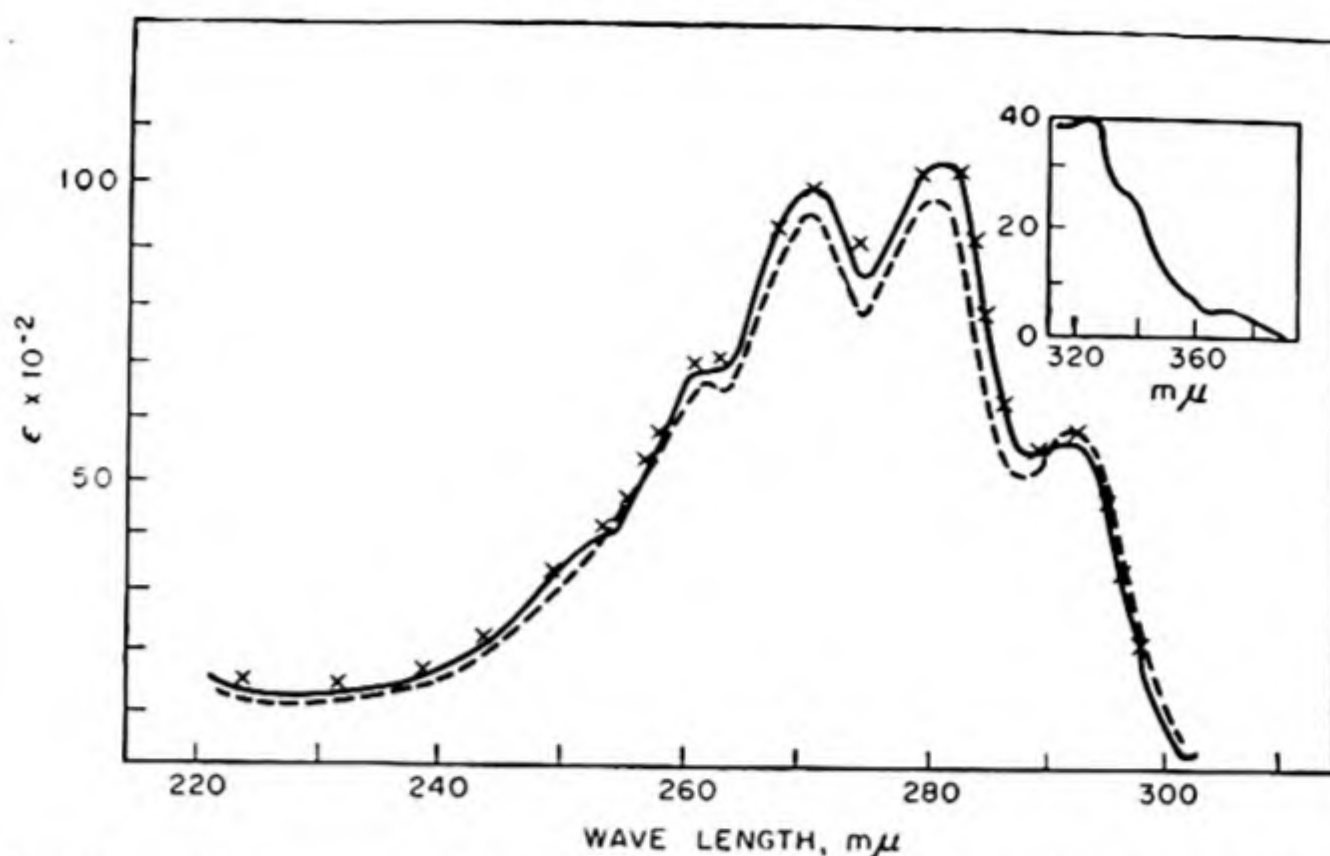


FIG. 5-18. Absorption spectrum of ergosterol. —, ergosterol in C<sub>2</sub>H<sub>5</sub>OH; ---, ergosterol in isooctane; X, 7-dehydrocholesterol. (After Hogness *et al.*, reproduced from *The Application of Absorption Spectra to the Study of Vitamins, Hormones and Coenzymes*, by R. A. Morton, 1942.)

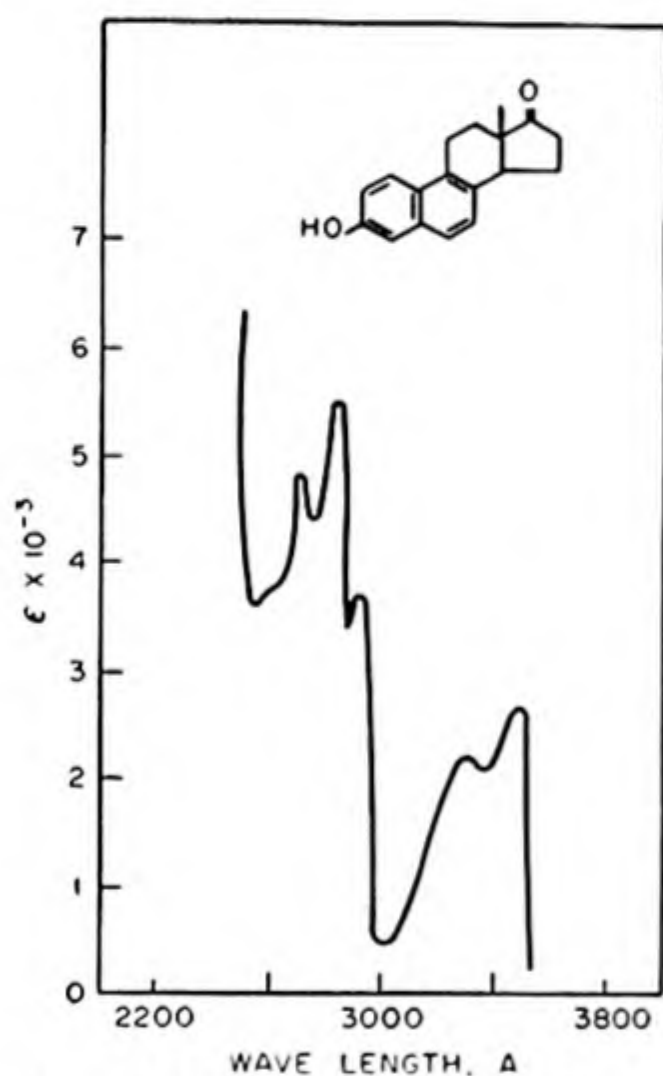


FIG. 5-19. Absorption spectrum of equilenin. (Jones, 1948.)

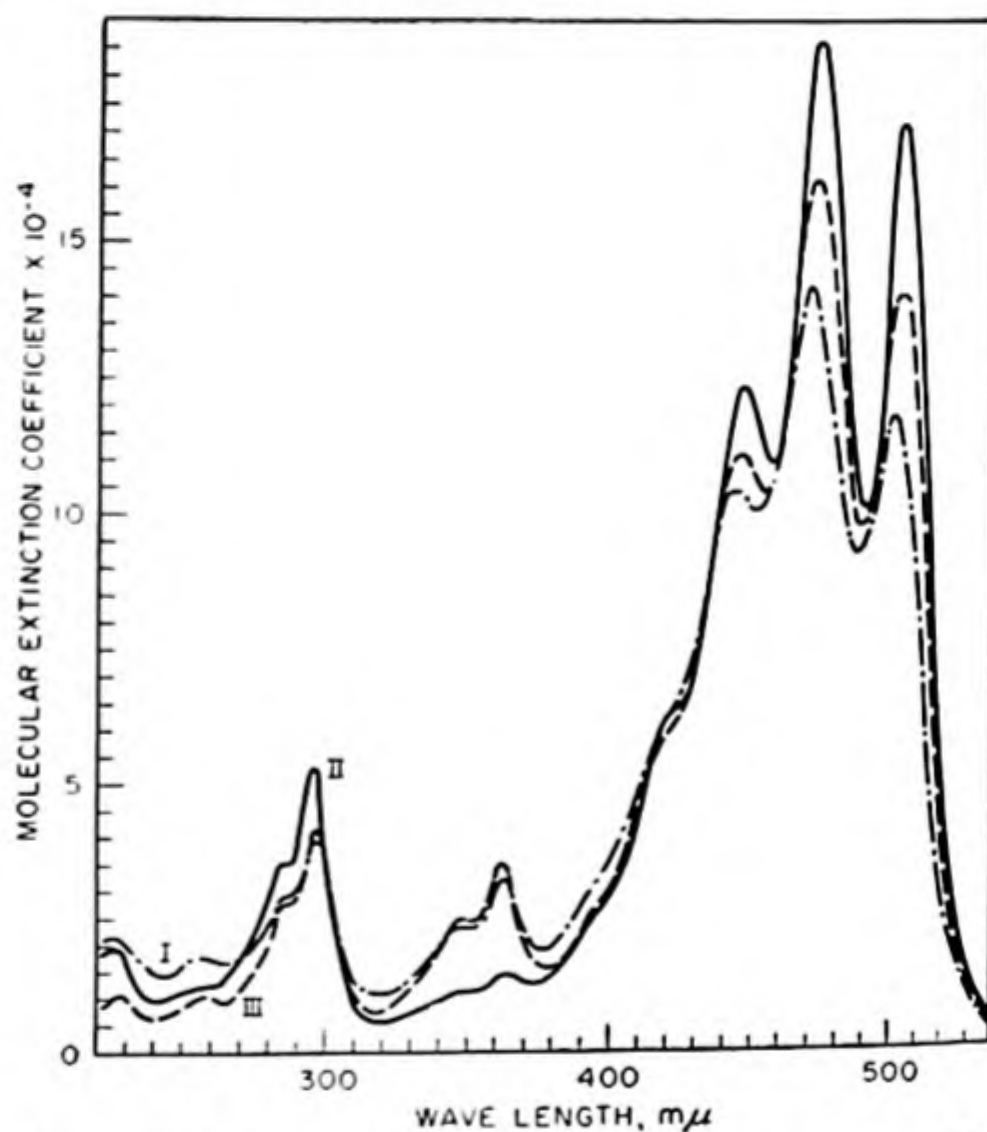


FIG. 5-20. Absorption spectrum of lycopene with varying degree of *cis-trans* isomerization. I, mixture of stereoisomers after iodine catalysis at room temperature in light; II, fresh solution of the all-*trans* compound; III, mixture of stereoisomers after refluxing in darkness for 45 min. (Zechmeister, 1944; copyright, 1944, by the Williams and Wilkins Company.)

Steroids with diene and triene chains, such as ergosterol (Fig. 5-18) and calciferol, will have strong absorption maxima in the region 2200–2900 Å (for calciferol,  $\lambda_{\text{max.}} = 2650$  Å, and  $\epsilon = 18,200$ ). Steroids that contain aromatic rings, such as the estrogens, will, in general, have strong absorption bands in the region 2400–2800 Å and may have absorption intensity well above 3000 Å if the aromatic grouping includes more than one ring, as in equilenin (Fig. 5-19) (Morton, 1942; Jones, 1948).

### CAROTENOIDS

The long conjugated double-bond chains of the carotenoid compounds give rise to from one to three ultraviolet absorption bands at varying positions (Karrer and Jucker, 1948).

One band is usually found in the region 260–320 m $\mu$ . Absorption in the region 320–380 m $\mu$  has been demonstrated to be dependent on the number and position of *cis*-configurations in the chain (Fig. 5-20) (Zechmeister, 1944). These ultraviolet bands

$$(\epsilon = 10,000\text{--}50,000)$$

are considerably weaker than the intense set of three bands commonly found in the visible absorption spectra of these compounds ( $\epsilon = 50,000\text{--}200,000$ ).

### PORPHYRINS

The intense Soret absorption band of the porphyrins is found in the near ultraviolet in the simpler members of the group, such as porphyrin itself (Fig. 5-21). With increasing substitution of the tetrapyrrole ring, this band moves into the visible-spectrum region. A number of shoulders or weak maxima are usually

to be found on the descending short wave limb of this peak. Porphyrins usually show a minimum of absorption in the region 2500–3000 Å and then increasing absorption again at wave lengths <2500 Å (Pruckner and Stern, 1937; Theorell, 1947; Holden, 1941).

The Soret band of the dihydroporphyrines, such as the chlorophylls, is

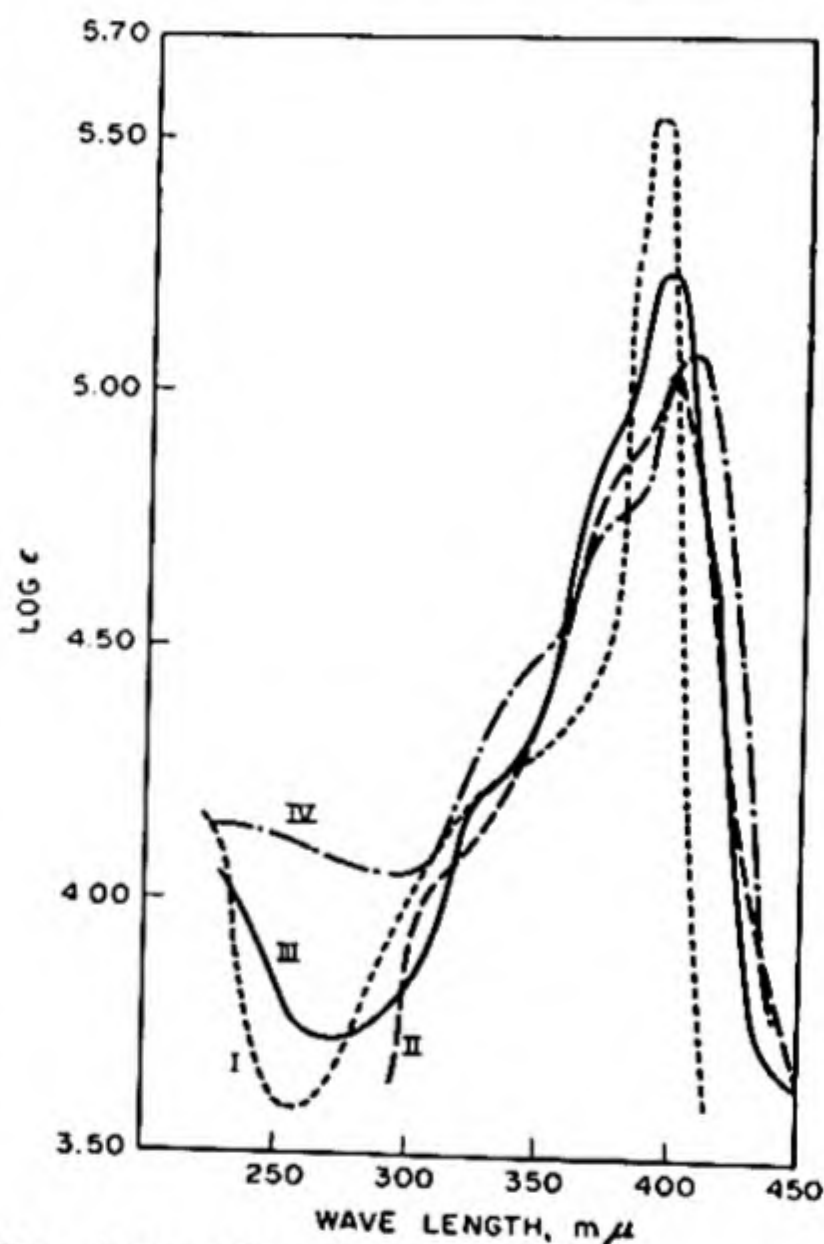


FIG. 5-21. Ultraviolet absorption spectra of several porphyrins in dioxane. I, porphyrin; II, aetioporphyrin; III, coproporphyrin-II-tetramethyl ester; IV, rhodoporphyrin-XV-dimethyl ester. (Pruckner and Stern, 1937.)



moved well out into the visible region (Granick and Gilder, 1947). The chlorophylls show several minor absorption peaks in the spectral region 2800–3800 Å (Fig. 5-22). Chlorophylls a and b differ notably in their absorption at about 3800 Å (Harris and Zscheile, 1943).

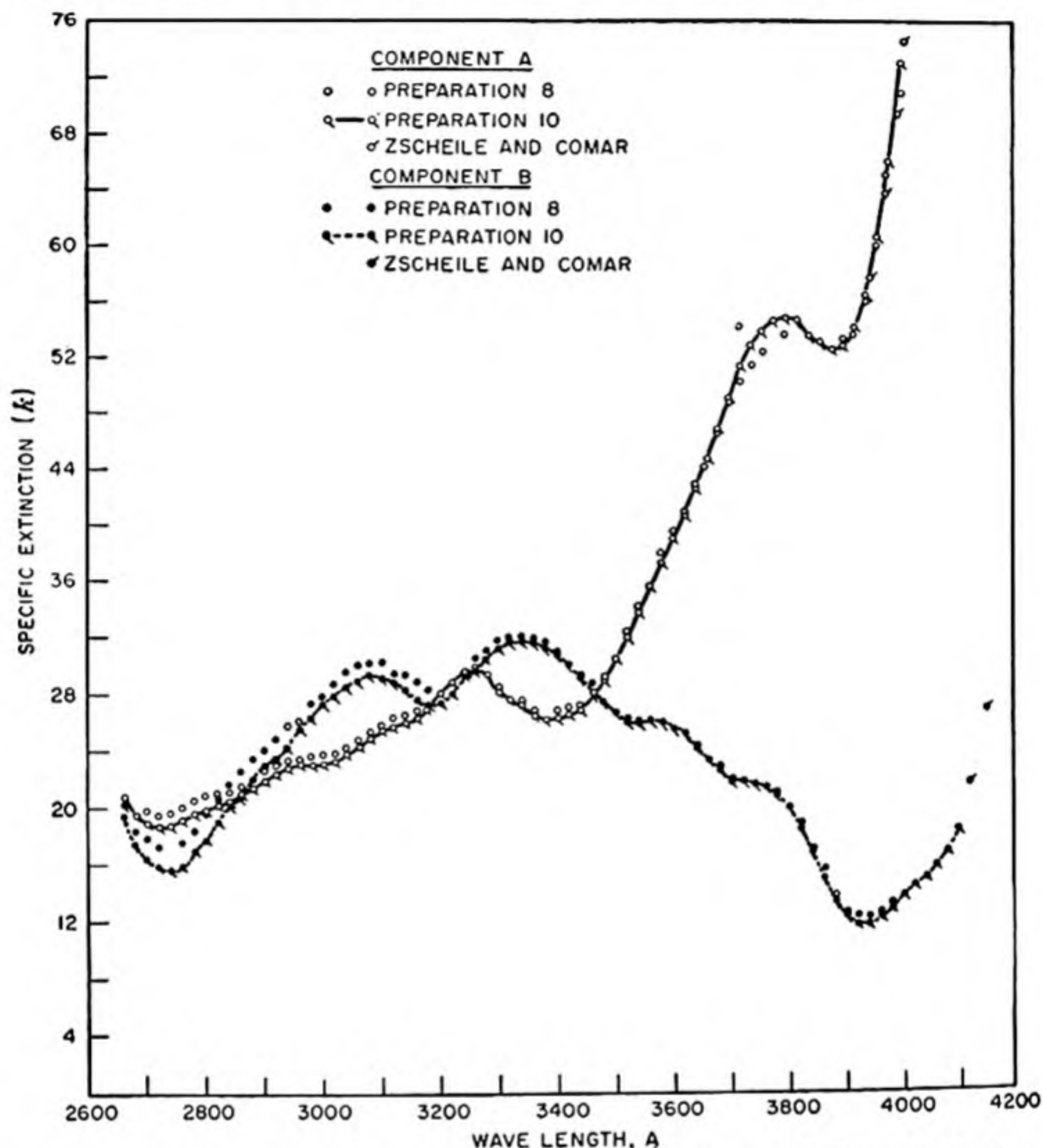


FIG. 5-22. Ultraviolet absorption spectra of chlorophylls A and B in ethyl ether solution. (Harris and Zscheile, 1943; reproduced from the *Botanical Gazette*, published by the University of Chicago Press.)

### FLAVINS

As with the porphyrins, more attention has been devoted to the visible absorption band of the flavins than to their ultraviolet spectra, which do, however, possess strong bands at 223, 265, and 370  $m\mu$ , as well as the visible band at 445  $m\mu$  (Fig. 5-23) (Warburg and Christian, 1938; Morton, 1942; Daglish *et al.*, 1943).

### PTERINS

The renewed interest in the pterins has focused attention on their ultraviolet absorption spectra, which generally contain one strong band in

the region 2400–2800 Å ( $\epsilon = 20,000$ ) and a second weaker band at 3400–3800 Å (Fig. 5-24) (Jacobson and Simpson, 1946; Totter, 1944; Mowat *et al.*, 1948; Hitchings and Elion, 1949; Cain *et al.*, 1948; Elion *et al.*, 1950).

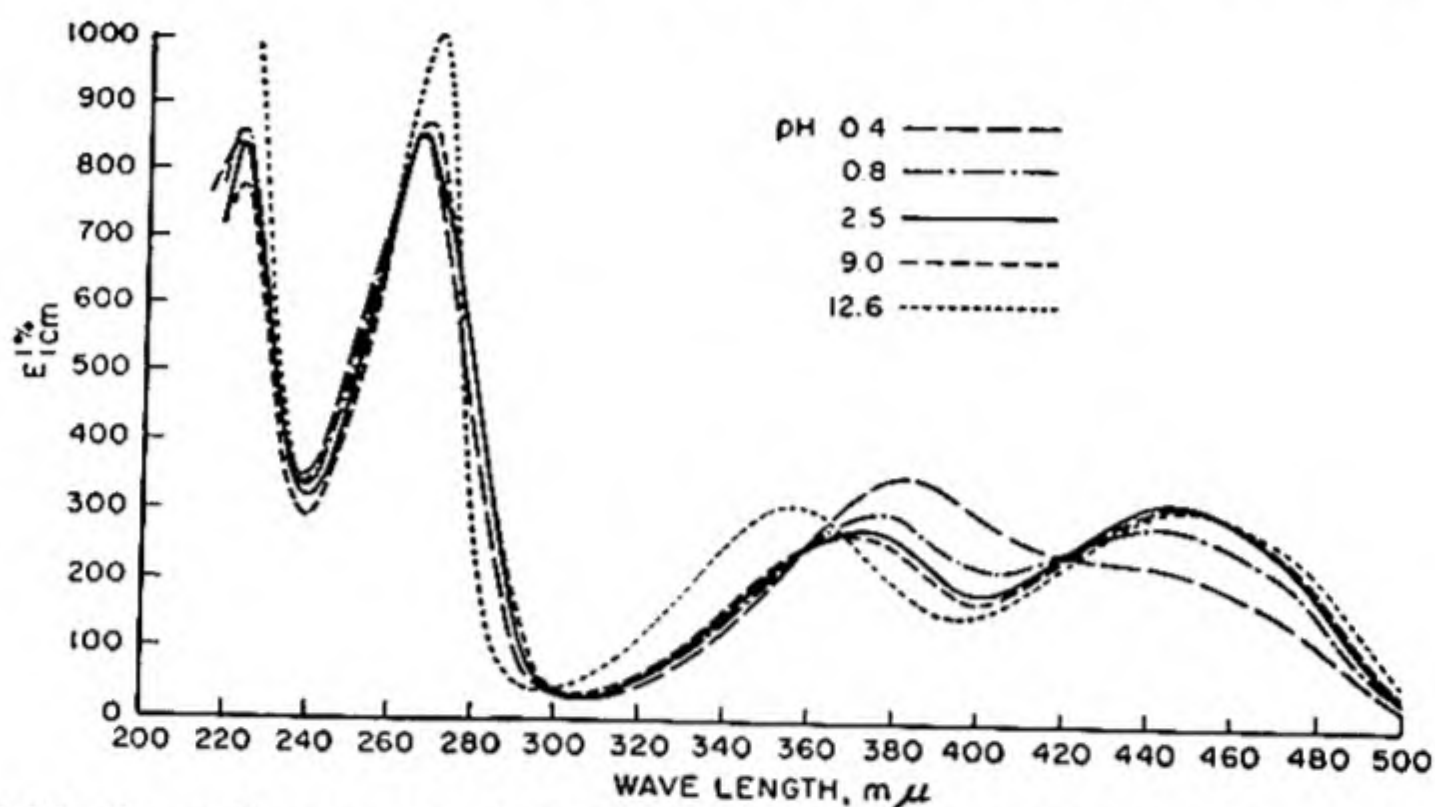


FIG. 5-23. Absorption spectra of riboflavin in solutions at different pH values. (Daglish *et al.*, 1948.)

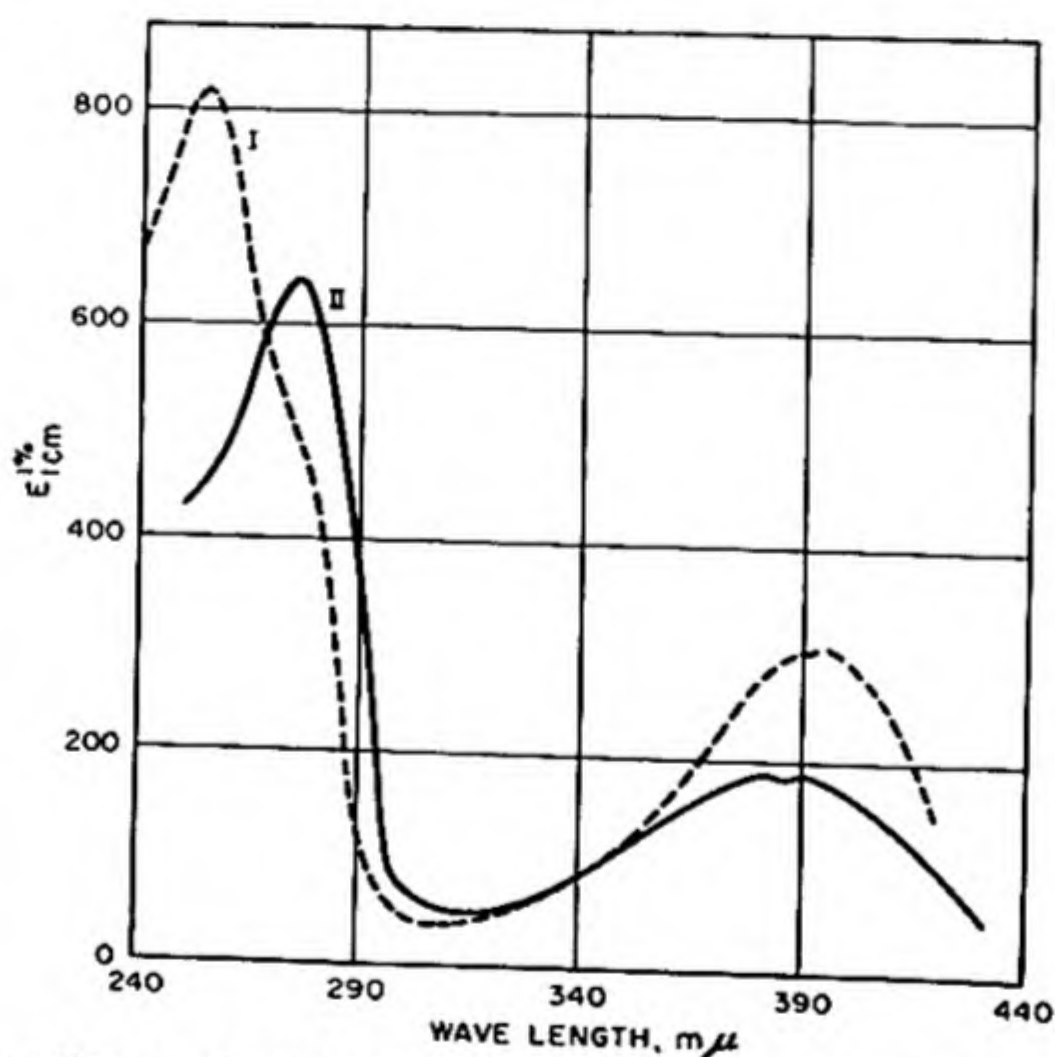


FIG. 5-24. Absorption spectrum of xanthopterin. I, in 0.1 *N* sodium hydroxide, 50.1 mg/liter; II, in glacial acetic acid, 49.4 mg/liter. (Totter, 1944.)

### VITAMINS

Reduced nicotinamide iodomethylate has a strong absorption band at 345  $m\mu$  which disappears upon oxidation (Karrer and Warburg, 1936;



Karrer *et al.*, 1936) and may then be restored by reduction. The disappearance and reappearance of this band in the spectra of coenzymes I and II (Fig. 5-25) (Euler *et al.*, 1936; Warburg *et al.*, 1935; Horecker and Kornberg, 1948) have been made the basis of elegant studies of the respiratory and fermentative enzymes by Warburg (1949).

Pyridoxine in neutral solution has absorption bands with maxima at 330  $m\mu$  ( $\epsilon = 5500$ ) and 255  $m\mu$  ( $\epsilon = 2800$ ). In alkaline solution these

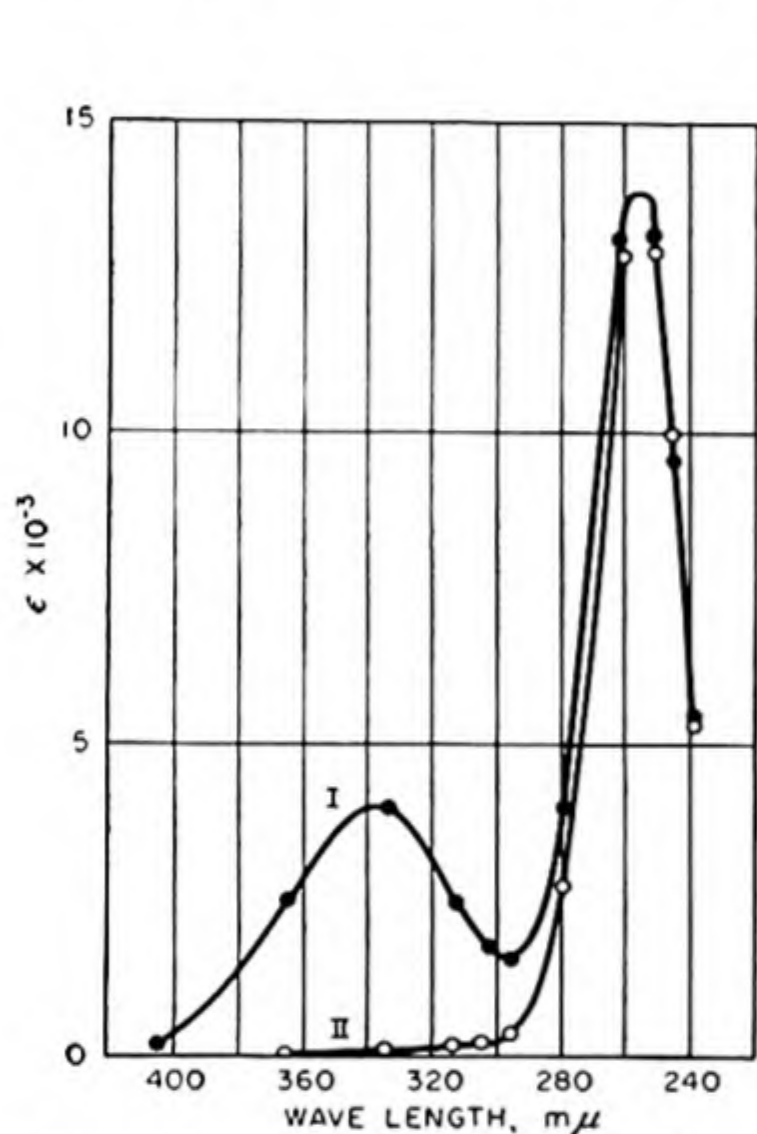


FIG. 5-25. Absorption spectra of reduced and oxidized cozymase. I, reduced cozymase; II, oxidized cozymase. (Euler *et al.*, 1936; reproduced from Hoppe-Seyler's *Zeitschrift für physiologische Chemie*.)

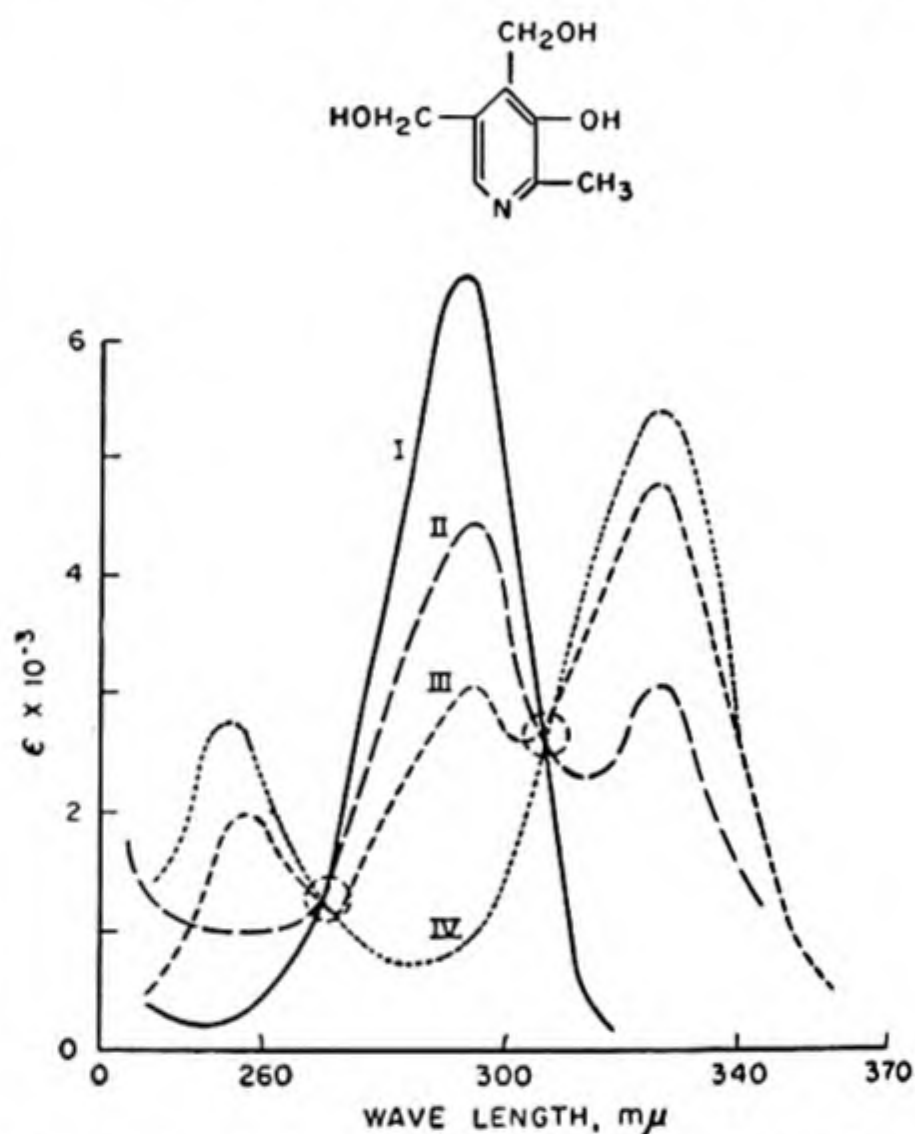


FIG. 5-26. Influence of pH on the absorption spectrum of pyridoxine (vitamin B<sub>6</sub>). I, pH 2; II, pH 4; III, pH 5; IV, pH 7. (From W. R. Brode, 1946, *The Absorption Spectra of Vitamins, Hormones, and Enzymes*. In, *Advances in Enzymology*, Vol. IV, F. F. Nord and C. H. Werkman, ed., copyright, 1944, by Interscience Publishers, Inc., New York.)

maxima are displaced toward shorter wave lengths, but in acid solution both maxima disappear and are replaced by a new peak at 292  $m\mu$  ( $\epsilon = 6600$ ) (Fig. 5-26) (Morton, 1942; Brode, 1946; Stiller *et al.*, 1939).

Vitamin B<sub>12</sub> has two strong ultraviolet absorption bands with maxima at 2780 Å ( $E_{1\text{cm}}^{1\%} = 115$ ) and 3610 Å ( $E_{1\text{cm}}^{1\%} = 204$ ) in addition to the weaker band in the visible spectrum at 5500 Å ( $E_{1\text{cm}}^{1\%} = 63$ ) (Brink *et al.*, 1949). Vitamins B<sub>12a</sub> and B<sub>12b</sub> have similar spectra (Kaczka *et al.*, 1949; Brockman *et al.*, 1950).

Ascorbic acid in neutral solution has a strong absorption band ( $\epsilon = 9300$ ) at 265  $m\mu$ , which shifts to 245  $m\mu$  in acid (Morton, 1942).

The presence of this band has at times been mistaken as an indication of the presence of nucleic acid (Strait *et al.*, 1947). Confirmation of the presence of ascorbic acid can be obtained by disappearance of the band upon oxidation, which can be induced by simply raising the pH above 10.

Vitamin E ( $\alpha$ -tocopherol) has a distinct absorption maximum at 2940 Å ( $\epsilon = 3200$ ). The  $\beta$ - and  $\gamma$ -tocopherols have similar spectra (Smith, 1940).

Vitamin K<sub>1</sub> has a strong, five-peaked absorption band in the region 2400–2800 Å and a weaker broad secondary maximum at 320 m $\mu$  (Fig. 5-27). The peaks of the former band occur at 239, 243, 249, 260, and 270 m $\mu$ , and for the 249-m $\mu$  peak,  $\epsilon = 19,600$  (Ewing *et al.*, 1943). Vita-

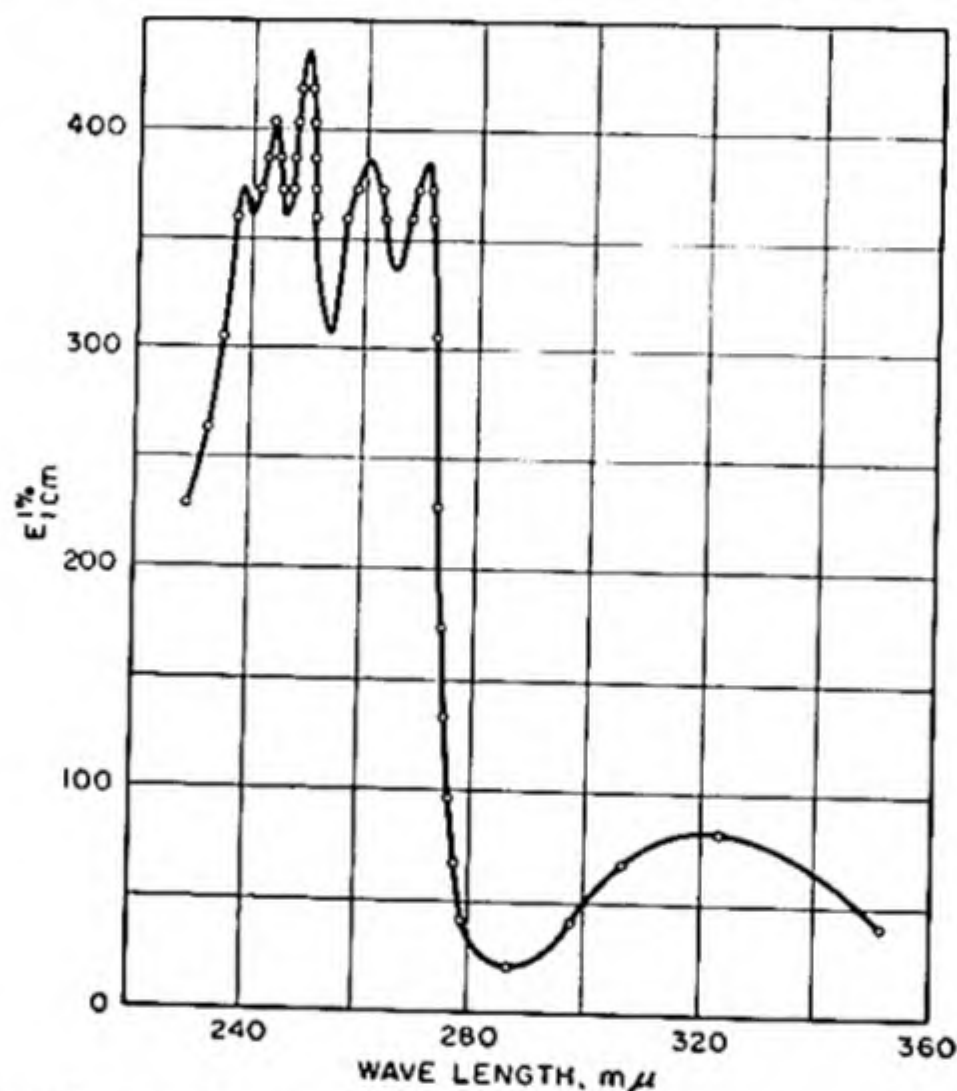


FIG. 5-27. Absorption spectrum of vitamin K<sub>1</sub> in hexane. (Ewing *et al.*, 1943.)

min K<sub>2</sub> has a similar, but slightly weaker, absorption spectrum (Ewing *et al.*, 1939).

### PLANT PIGMENTS

The common plant pigments such as the anthocyanins and flavones have strong ultraviolet absorption bands. In acid solution, all anthocyanidins and anthocyanins have one or two strong absorption maxima in the region 2650–2800 Å ( $\epsilon = 10,000$ –20,000) (Fig. 5-28). Some have additional bands at  $\sim 2450$  and  $\sim 3300$  Å. In basic solution these ultraviolet absorption bands, as well as the visible bands responsible for the color of these pigments, are displaced a few hundred Angstroms toward the longer wave lengths (Schou, 1927; Hayashi, 1934, 1936).

The flavones have two ultraviolet absorption bands at 2500 and 3000 Å ( $\epsilon \approx 10,000$ ). Hydrogenation of the chromene ring, as in the flavonones,



moves the position of the longer wave band to 3200 Å, without affecting the 2500 Å band. Addition of a 3-hydroxy group to flavone, as in the flavonols causes the appearance of an additional band at 3350–3400 Å (Fig. 5-29). Hydroxy or methoxy side groups in various positions may

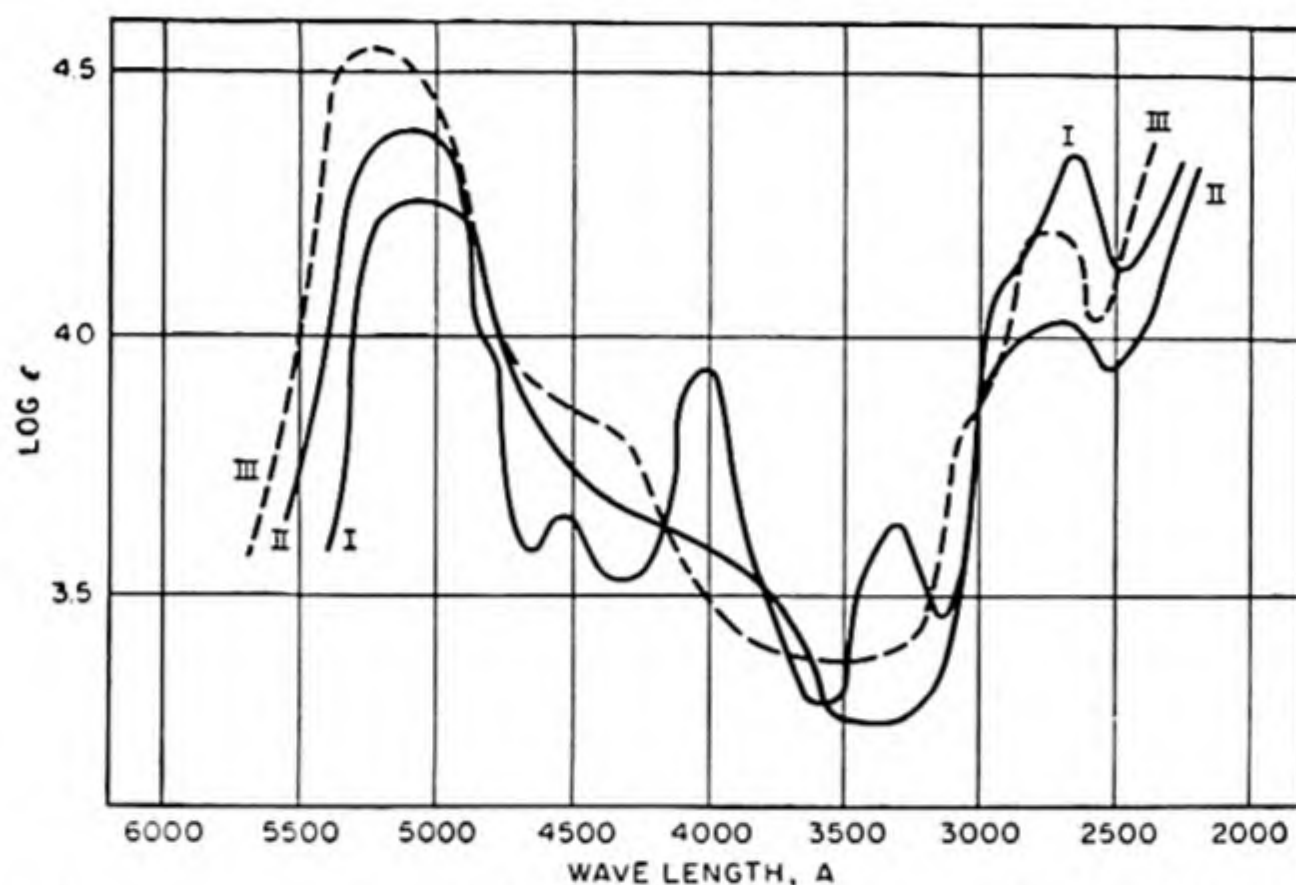


FIG. 5-28. Absorption spectra of I, pelargonidin; II, cyanidin; and III, delphinidin. (Schou, 1927.)

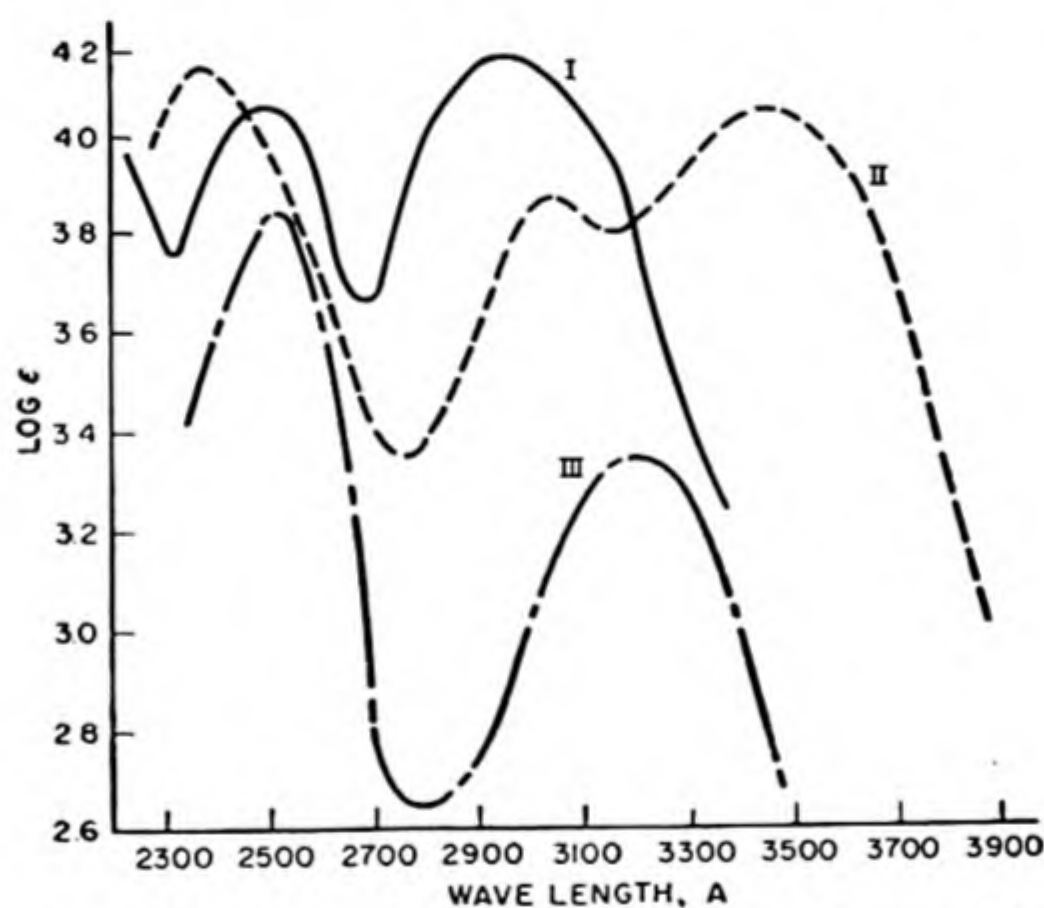


FIG. 5-29. Absorption spectra of I, flavone; II, 3-oxy-flavone (flavonol); and III, flavonone. (Skarzynski, 1939.)

shift these maxima by as much as 100–200 Å (Aronoff, 1940; Skarzynski, 1939).

The catechins, which occur widely in woods and leaves, may be regarded as partially reduced anthocyanidins. The reduction has destroyed the extensive conjugation, and the absorption of catechins may

be regarded as the sum of the absorption of the phloroglucinol nucleus and the polyphenolic residue. Catechins generally have absorption maxima in the region 2700–2800 Å ( $\epsilon = 1000$ –3000). Secondary maxima may appear about 2150–2200 Å (Bradfield and Penney, 1948; Morton and Sawires, 1940; Klingstedt, 1922).

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## CHAPTER 6

# A Critique of Cytochemical Methods

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*Introduction. Laws of absorption. Localization of substances in cells: Preservation of intracellular substances in situ—Nucleic acid staining and tests—Protein staining and tests—Ultraviolet absorption of nucleic acids and proteins. Quantitative microscopical methods: Visual comparison—Photometric technique—Some errors of quantitative microspectrophotometry—Quantitative applications, absolute and relative. References.*

### 1. INTRODUCTION

The bulk of the extensive researches in cytology has been aimed primarily at demonstrating the morphological features of the cell. By 1875 modern microscopes had become available which reached close to theoretical limits imposed by the properties of visible light, and within less than two decades the application of this tool, in conjunction with an increasing number of special microtechniques for preparing cells for examination, had demonstrated a wide diversity in intracellular morphology and many striking correlations of cell structure with physiology. From these studies the concept of the histological unit, the cell, emerged with increasing clarity (Figs. 6-1, 2A).

A similar morphological picture of most cell types could be drawn from the researches of three-quarters of a century of cytology. Such a description, of course, is completely unsatisfactory from the standpoint of intracellular biochemistry; indeed, it is merely an invitation to further research which might lead to more nearly complete understanding of the physiological processes by which such a cell can synthesize a secretory granule or can elaborate the material to duplicate itself. One obvious way in which to complete the picture is to attempt to determine the chemical composition of the cell. To a cytologist this problem emerges as one of demonstrating how the various substances, which biochemists obtain by such procedures as extraction from minced organs, are distributed among the various visible cellular constituents and in the apparently structureless material which fills spaces between the nuclei, mitochondria, granules, and other formed elements of the cell. Broadly regarded, this appears to be the field of what has come to be called



“cytochemistry,” and in this chapter an attempt will be made to describe and to evaluate critically some of the approaches to this problem of chemical organization of the cell.

## 2. LAWS OF ABSORPTION

Since cytological studies are necessarily carried out at high magnification with a compound microscope the preparation, a section or smear of an organ, is always examined by transmitted light. For this reason the

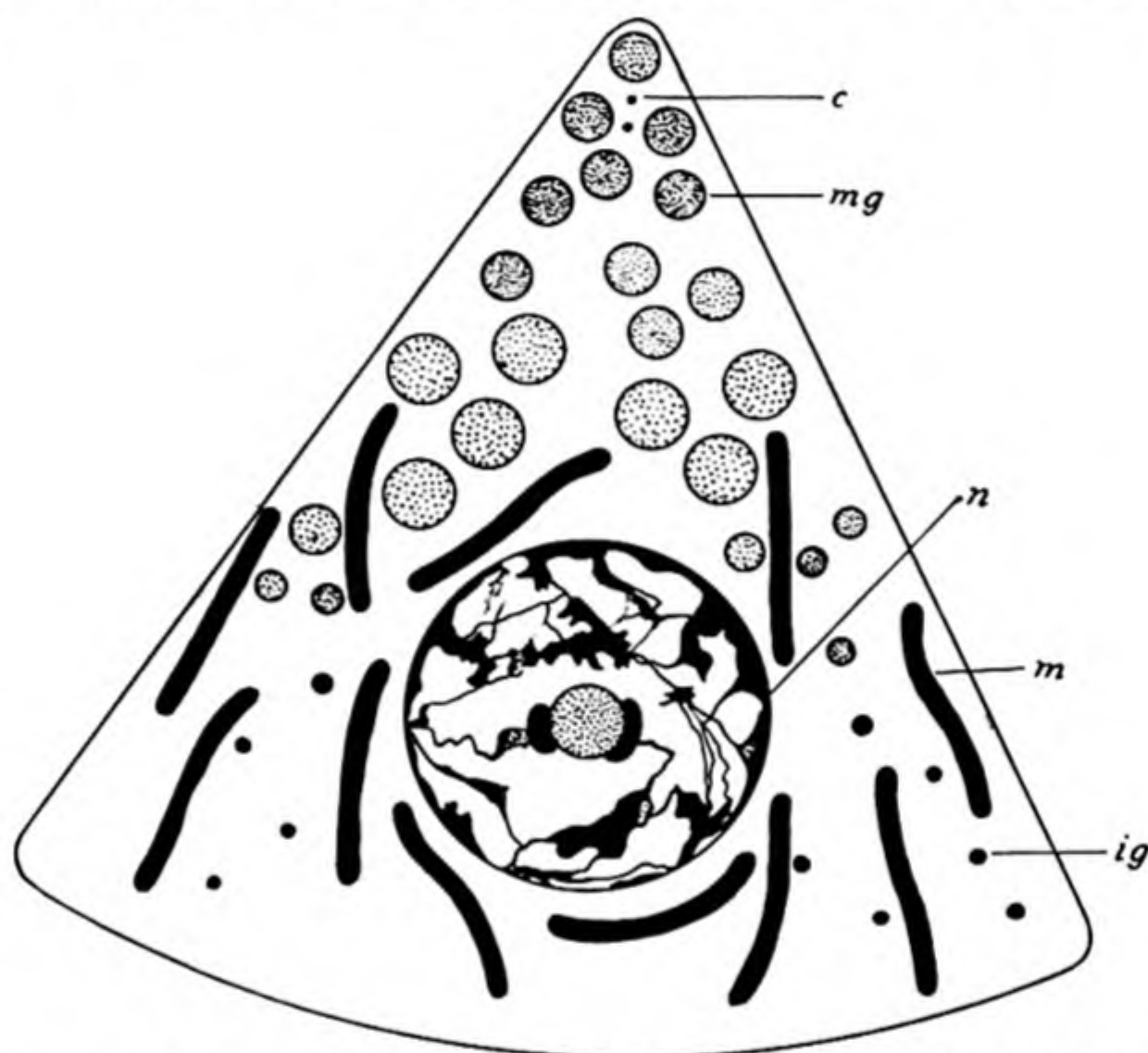


FIG. 6-1. Diagram of the structural features of a serous glandular cell at an early stage in restitution of the secretory granules. *c*, centriole; *mg*, mature zymogenic granule; *ig*, immature zymogenic granule; *n*, nucleus with masses of chromatin and a large spherical nucleolus; *m*, mitochondrion.

technique of cytological microscopy is readily adapted to photometric chemical analysis, in which the nature and amount of material may be determined from the spectral characteristics and intensity of the light which emerges from a semitransparent mass. This fraction of the light is said to be “transmitted”; that portion which entered the object but did not emerge is said to have been “absorbed.” Application of laws of absorption (see Chap. 5, this volume) not only make it possible to use on slides qualitative and quantitative methods of chemical analysis, but these laws also apply directly to the visual examination of cells since the visibility of a natural or artificial color in a cell depends on whether the colored object absorbs enough light to make it distinguishable from the surrounding nonabsorbing regions.

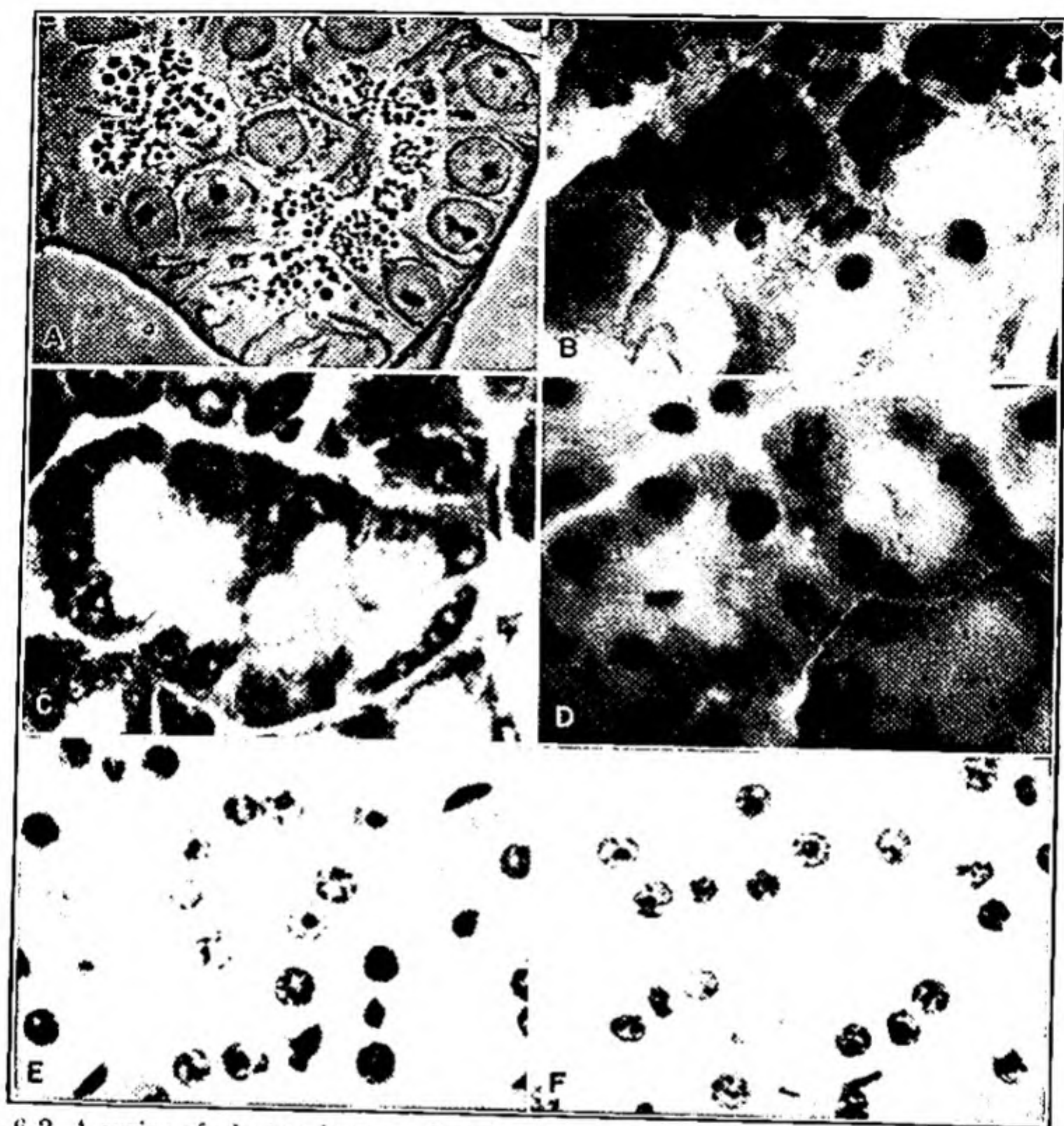


FIG. 6-2. A series of photomicrographs of pancreatic glandular cells, showing structure and the results of various techniques of localization of nucleic acids and proteins. (A) phase contrast, no stain or reaction; (B) Millon reaction for total protein; (C) nucleic acids stained specifically by basic dye, azure A; (D) ultraviolet photograph (254 m $\mu$ ) showing absorption in regions of nucleic acid concentration; (E) digested with ribonuclease before staining with azure A [compare with (C)]; (F) Feulgen's nuclear reaction for deoxypentose. (Pollister *et al.*, 1951.)

Inferences from the more general laws of relation of mass to absorption of radiant energy are conveniently summarized by some simple equations, in which the following symbols are used:

$I_0$  = light intensity (galvanometer reading) when no absorbing object is in the optical path (in cytological measurements, the reading through an empty part of the slide);

$I_x$  = intensity when the absorbing object or sample is in the optical pathway (in cytological measurements, the reading through the cell component);

$T$  = transmission,  $I_x/I_0$  ( $\% T = 100T$ );



- $E$  = extinction, also called optical density,  $\log_{10} 1/T$  or  $\log_{10} I_0/I_x$ ;  
 $\lambda$  = wave length;  
 $E_\lambda$  = extinction at a given wave length, e.g.,  $E_{550 \text{ m}\mu}$ ;  
 $(E_\lambda)_a^n$  = extinction for unit amount per unit area;  
 $c$  = concentration;  
 $l$  = length of absorbing pathway;  
 $A$  = area of absorbing mass in plane perpendicular to the absorbing pathway;  
 $V$  = volume of absorbing mass;  
 $k$  = specific extinction, e.g.,  $(E_\lambda)_{1.0 \text{ cm}}^{1\%}$ .

Beer's law deals with the relation of light loss to concentration ( $c$ ); Lambert's law deals with the relation of light loss to absorption path ( $l$ ). A useful simple equation which expresses the fact that extinction is directly proportional to the number of specifically absorbing chromophores ( $k$ ) in the absorbing path, as determined by concentration and thickness is

$$E = kcl.$$

In most routine quantitative photometric chemical analysis the sample is in a carefully measured cuvette or absorption cell (generally 1 cm thick). The unknown concentration ( $c_x$ ) may be computed in terms of the extinction of a standard of unit thickness and concentration, e.g.,  $(E_\lambda)_{1.0 \text{ cm}}^{1\%}$ , where  $E_x$  is the extinction of the unknown from the following formula.

$$C_x \% = \frac{(E_\lambda)_x}{(E_\lambda)_{1.0 \text{ cm}}^{1\%}}.$$

In cytological preparations the thickness is always but a few microns. When this thickness has been measured, computation from a standard extinction must take into account the relative thickness. An equation by which, assuming the validity of Beer's law over the concentration range of which the two measurements are the extremes, the percentage concentration in a cytological preparation may be computed from the standard  $(E_\lambda)_{1.0 \text{ cm}}^{1\%}$  is

$$c_x \% = \frac{10,000 (E_\lambda)_x}{l_x (E_\lambda)_{1.0 \text{ cm}}^{1\%}}$$

where  $l_x$  is the thickness of the cytological structure, in microns.

Instead of using the standards obtained in the cuvettes of a colorimeter or spectrophotometer, it is more convenient to compute special cytological standards. Most useful is  $E_a$  computed as the extinction for  $10^{-10}$  mg/ $\mu^2$ ,  $(E_\lambda)_{\mu^2}^{10^{-10} \text{ mg}}$ , in which Caspersson has expressed the results of his quantitative cell analyses. In a thickness of 1  $\mu$  this is equivalent to 10 per cent, to 100 mg/cc, or to  $10^{-10}$  mg/ $\mu^3$ . This standard has been com-

puted from *in vitro* data for a number of naturally colored substances commonly found within cells and for some specific stains and tests for proteins and nucleic acids (columns 1, 2, and 3, Tables 6-1, 2). The sources of the values from which computations were carried out are indicated in column 5.<sup>1</sup>

These values in Tables 6-1 and 2 are not claimed to be necessarily close approximations of any physical constants of the intracellular substance. They are almost certainly subject to considerable revision as more is learned of the effects on absorption of high concentrations and of special intermolecular associations within the cell (see p. 215 and Chaps. 1 and 5, this volume). In the meantime, they are useful relative values from which the possibilities of seeing, or measuring photometrically, an intracellular substance under the microscope can readily be estimated; they are likewise the only method of translating results of intracellular absorption measurements into the familiar values of chemical analysis, and in radiation experiments they can serve as the basis for an approximate estimate of the amount of energy absorbed per cell or cell part. Since the errors introduced by high concentrations and other special conditions within the cell tend, as a rule, to reduce the specific extinctions, it seems fairly safe to assume that the values in column 3 are maximal and those in column 4 are minimal. The great usefulness of the cytological standard,  $(E_\lambda)_a$ , arises from the fact that of itself extinction is a direct measure of the number of molecules in the absorbing path and can be used as such when neither concentration nor thickness of the absorbing area is known. From the standard  $(E_\lambda)_{\mu^2}^{10^{-10}\text{mg}}$  the amount per total area of the part of the cell measured ( $A$ ) can be computed simply as  $(E_\lambda)_a \cdot A$  ( $A$  being measured in square microns). Likewise, because extinction times area is the equivalent of amount, it can be used in simple arithmetical computations to compare compositions of cytological objects in purely arbitrary amounts (see Swift, 1950).

So far we have been considering photometric methods with measurement in a more or less restricted spectral region, a procedure which, somewhat paradoxically, is often called colorimetry. When measurements are made at many wave lengths, a picture is obtained of the effect of the absorbing substance on the light, which expresses in objective data the phenomena which cause the visual sensation of color. Such data are often plotted as absorption curves, with some measure of relative light

<sup>1</sup> The cytological standard,  $(E_\lambda)_{\mu^2}^{10^{-10}\text{mg}}$  is computed as follows, from a cuvette standard, e.g., for deoxyribonucleic acid (DNA) where  $(E_{254})_{1.0\text{cm}}^{1.0\text{mg}/\text{cm}^2}$  is 20. It is given that 20 is the  $E_{254}$  of  $1\mu^2$  of a standard solution in a thickness of  $1.0\text{cm}$ ,  $10^4\mu$  (for extinction depends on thickness and concentration and is independent of area). Each cubic centimeter of cuvette standard contains  $1.0\text{mg}$  of DNA;  $1\text{cc}$  is  $10^{12}\mu^3$ , and the volume of a mass which is  $1\mu^2$  in area and  $1\text{cm}$  thick is  $10^4\mu^3$ . This volume of the standard solution then contains  $10^4/10^{12}$ , or  $10^{-8}$ ,  $\text{mg}$  of DNA. Therefore,  $(E_{254})_{\mu^2}^{10^{-10}\text{mg}}$  is  $20/10^8$  or  $0.200$  (Table 6-1).



TABLE 6-1. NATURAL ABSORPTION OF SUBSTANCES OCCURRING IN CELLS

Substance	Wave length, $m\mu$	$(E_{\lambda})_{\mu^2}^{10^{-10} \text{ mg}}$	Concentration (%) in $1 \mu$ to give $E_{\lambda}$ of 0.030	Reference
Deoxyribonucleic acid . .	254	0.200	1.5	Ris (1947)
Deoxyribonucleic acid . .	260	0.220	1.4	Caspersson (1940a)
Deoxyribonucleic acid . .	275	0.180	1.7	Caspersson (1940a)
Ribonucleic acid . . . . .	260	0.230	1.3	Thorell (1947)
Tryptophane . . . . .	275	0.270	1.1	Fig. 6-21; Caspersson (1940a)
Tyrosine (acid) . . . . .	275	0.065	4.6	Fig. 6-21; Caspersson (1940a)
Tyrosine (alkali) . . . . .	290	0.090	3.3	Fig. 6-21; Caspersson (1940a)
Serum albumen . . . . .	275	0.006	50.0	Fig. 6-5
Serum albumen . . . . .	260	0.0016	Not detectable	Fig. 6-5
Ascorbic acid . . . . .	265	0.355	0.85	Stearns (1950)
Thiamine . . . . .	232	0.312	0.96	Stearns (1950)
Riboflavin . . . . .	265	1.78	0.17	Stearns (1950)
Vitamin A . . . . .	324	1.82	0.16	Stearns (1950)
Oxyhemoglobin . . . . .	413	0.069	4.3	Thorell (1947)
Chlorophyll a . . . . .	665	2.90	0.10	Zscheile (1934)
Chlorophyll b . . . . .	640	1.22	0.25	Zscheile (1934)
Cytochrome c . . . . .	415	0.665	0.46	Dixon <i>et al.</i> (1931)

TABLE 6-2. ABSORPTION OF CYTOCHEMICAL STAINS AND TESTS

Substance	Wave length, $m\mu$	$(E_{\lambda})_{\mu^2}^{10^{-10} \text{ mg}}$	Concentration (%) in $1 \mu$ to give $E_{\lambda}$ of 0.030	Reference
Deoxyribonucleic acid, Feulgen reaction . . . .	546	0.200	1.5	Alfert, 1950
Tryosine, Millon reaction . . . . .	365	0.220	1.4	Pollister (1950)
Protein, Millon reaction (6.25% tyrosine)	365	0.014	21.0	Pollister (1950)
Protein, Millon reaction (6.25% tyrosine)	490	0.007	43.8	Pollister (1950)
Fast green, pH 2.0 . . . .	625	1.6	0.19	Bryan (1951)
Egg albumen, fast green	625	0.568	0.53	Computed from Fraenkel-Conrat and Cooper (1944); Bryan, 1951

loss on the vertical axis, and wave length or frequency on the horizontal axis. The shape of the absorption curve is of qualitative value in identifying the specific absorbing atomic configuration, the chromophore. When a sample contains two or more nonreacting absorbing substances, the compound absorption curve results from addition of the individual components. If the curves of the chromophores are considerably different in absorption coefficient at some wave lengths, the compound curve can be analyzed into the individual curves of its components by solving simultaneous equations (Stearns, 1950).

As Tables 6-1 and 2 indicate, in order to be detectable in the cell by absorption (i.e., by contrast), substances must reach a concentration many thousand times that which is sufficient for analysis in an absorption cuvette. This imposes a fundamental limitation on the whole method of interpreting the chemical composition of cellular components from visual microscopical appearance or, indeed, from the most careful microscopical absorption measurements with an objective photometer. Very small quantities can be detected, as little as  $10^{-14}$  g in a single small granule, which may be perhaps no more than a millionth of the entire cell volume. Therefore, in one sense, these are very "sensitive" techniques in the vocabulary of the microchemist (see Benedetti-Pichler and Rachele, 1940). But, as a means of demonstrating a complete picture of distribution of a substance within the cell, these microscopic methods are deplorably inadequate. Obviously, if the concentration within a small granule just reaches the threshold for detection (which experience shows to be roughly equivalent to an extinction of 0.03 or about 7 per cent absorption; see Tables 6-1 and 2, column 4), then outside this spot of high local concentration there can be a relatively enormous amount of substance which is below the detectable absorption or contrast. It is a simple matter to compute in any cell, from data like those of Tables 6-1 and 2 and the volume relations within the cell, the maximum possible amount of substance that could escape visualization or measurement. Conclusions about localization and distribution of intracellular substances from microscopical data must always take into account this interrelation between absorption and intracellular geometry. It must be emphasized that microscopic methods alone can prove neither the exclusive localization of a substance within a small intracellular structure nor the complete absence of a substance from any part of the cell.<sup>2</sup> Such conclusions can come only from a combination of methods of chemical analysis of cell isolates and cytology, as pointed out by Pollister, Himes, and Ornstein (1951).

<sup>2</sup> One escape from this limitation on microscopic detection and estimation lies in developing stains which are fluorescent and tests based on fluorescence. Since fluorescence is seen or measured as total intensity against a dark field, i.e., zero intensity (instead of by subtraction from a field of high intensity), the dye or color reaction can be detected readily in concentrations as low as one one-thousandth of the minimum



### 3. LOCALIZATION OF SUBSTANCES IN CELLS

It must not be supposed that an awareness of the difficulties just summarized has operated to inhibit the development of a chemical cytology. Quite the contrary has been the case; from the earliest days of cytology there have been attempts to supplement the morphological descriptions of cells by some idea of the chemical composition.

A few substances such as hemoglobin or chlorophyll are visible in the living cell because of the natural color, but in most cases a substance can become visible only because of a color reaction carried out on a microscopic slide. To be useful cytochemically such a test or stain must fulfill certain criteria:

1. It must be possible to carry out the test under conditions which will not seriously distort the cell morphology, a requirement which excludes a great many of the color reactions of analytical chemistry.

2. The specificity of the reaction must be known from data obtained *in vitro*.

3. The reaction must be one which will proceed without interference in the presence of large amounts of proteins, and often in the presence of nucleic acids or lipids.

#### 3-1. PRESERVATION OF INTRACELLULAR SUBSTANCES *IN SITU*

Lison (1936) has considered critically many of these so-called "histochemical" techniques; additional ones are briefly mentioned by Glick (1949). The microchemical tests for inorganic ions, such as ferric, chloride, and phosphate, and for smaller organic molecules, such as uric acid and oxalic acid, are for the most part closely analogous to those of microchemistry and cause the appearance on the slide of a colored precipitate or crystals only when the reacting group is in solution. For cytological studies this means that the group will not react when it is a part of a large molecule, such as that of protein, but only after it has been split off. These tests can undoubtedly be interpreted as an indication that the reacting substance is present in the section of tissue, but this is but a poor imitation of the precise conclusions which are possible from the methods of analysis of tissue masses (Hogeboom, 1951) or from refinement of microchemical methods to reach down to the level of a single cell (Norberg, 1942). For cytological studies the intracellular localization is most important, and there are good reasons to question the validity of methods of microscopical demonstration of small diffusible chemical

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detectable by absorption. The possibilities have not been widely explored, but a fluorescent Schiff reagent has been developed and found to demonstrate marked aldehyde (plasmal?) reaction in cells where the test appeared completely negative by absorption contrast technique (L. Ornstein, unpublished). Fluorescent cytological techniques also offer one possibility of avoiding the distributional error (p. 235).

entities by color reactions. Within the dimensions of the cell, diffusion—a slow process at the macroscopic level—is practically instantaneous, and there is every reason to suppose that extensive redistribution takes place, either as tissue is being fixed or as the test is carried out on the tissue. This is particularly misleading because the precipitates tend to be adsorbed on the extensive internal surfaces of the denser parts of the cell such as nuclei, myofibrillae, or the thick distal borders of some epithelial cells. Thus many early observers erroneously reported that the nucleus contained considerable iron, an element in which it is actually notably deficient. The nucleus was repeatedly described as the site of the enzyme alkaline phosphatase, since phosphate split off from a substrate, glycerophosphate, appeared as an intranuclear precipitate, but Jacoby and Martin (1949) have demonstrated that this is largely a secondary accumulation of the phosphate (see also Novikoff, 1951, 1952).

Redistribution during the fixation process is effectively prevented by the freeze-dry method of preparing sections of tissue, which was suggested by Altmann (1890) and elaborated by Gersh (1932), Hoerr (1936), Simpson (1941), and others. In this technique fresh tissue is quickly frozen at such low temperatures ( $-190^{\circ}\text{C}$ ) that ice crystals do not form (the water appears rather to be practically vitrified). Tissue is then dehydrated at low pressure and temperature and is finally embedded in paraffin and sectioned. Up to this stage, it is generally agreed that little redistribution of intracellular chemical constituents can have taken place, and two excellent methods of elementary chemical analysis apparently can be carried out without producing any essential change in this distribution. The paraffin sections may be burned in an electric furnace (microincinerated; Policard, 1923; Scott, 1943), and the appearance and amount of the ash indicate the distribution of the mineral elements in the cells (e.g., iron is a yellow to red ash, silicates are crystalline, and calcium and magnesium are amorphous and dense white ashes.) In another method the paraffin is removed from the section, and the tissue is dried and subjected *in vacuo* to X-ray absorption analysis (Engstrom, 1946, 1950). Although it involves immense technical difficulties, the latter appears to be an extremely promising approach since it offers the possibility both of determination of cell mass from polychromatic X-ray absorption data and of analysis for a large number of individual elements from absorption of monochromatic X rays in the wave length range 2–50 Å.

The distribution in frozen-dried sections would also be expected to remain essentially unchanged, except for lipoidal constituents, if the paraffin is removed—for instance, by chloroform—and the section kept in nonaqueous solvents such as alcohol and glycerin. This procedure has been recommended in preparing material for ultraviolet absorption studies of intracellular proteins (Caspersson, 1947) principally because



the optical conditions within the tissue are then more favorable for these measurements. This advantage would appear to be somewhat offset by the confusion which is introduced into the absorption picture by the probable presence, in unextracted material, of many absorbing compounds of low molecular weight, a situation like that which makes the results of ultraviolet absorption study of living cells so inconclusive.

When precipitation or a color reaction on the microscopic section necessarily involves use of aqueous reagents, then the frozen-dried sections must be rehydrated. This in effect appears to eliminate about every advantage of the freeze-drying technique because extensive redistribution can take place at once and can continue during any subsequent steps of the technique (Hoerr, 1943). Unless these technical difficulties can be overcome, the sound view seems to be to admit that, except for microincineration and X-ray absorption, precise intracellular localization is practically limited to substances of high molecular weight, which diffuse slowly and are easily converted into relatively insoluble masses, and to the use of tests which demonstrate the smaller chemical groupings when they are parts of these large molecules. Two substances of this character, proteins and nucleic acids, bulk large in the composition of all cells. Indeed, it is easy to see that, if these two constituents are removed, as can be done with enzymes, the remainder is but an unrecognizable ghost of a cell. As a matter of fact, the whole concept of the fixed cell is mainly that of a nucleoprotein mass. Except for some of the special mitochondrial methods which preserve lipids, the vast majority of cellular studies have been made on cells fixed in strongly acid fluids which, while precipitating admirably proteins and nucleic acids, at the same time must wash out smaller unattached cellular constituents, organic or inorganic, to such an extent that the residue can hardly be great enough to be cytologically detectable (see Pollister, 1952a). This predominance of nucleoprotein in cellular composition has always been so obvious that it is possible to overlook its significance to cytochemistry. For example, in Lison's *Histochemie animale* (1936) this point is not stressed, and there is a distinct impression that the primary concern of histochemists should be the localization of simple chemicals such as iron and amino acids.

Whatever the special objectives of a study of intracellular localization, a very obvious fact about cellular composition is that, in all cells, proteins and nucleic acids occur in high enough concentration so that tests for them fall within the visible, or measurable, range, and consequently techniques for nucleoprotein demonstration are applicable to a great variety of problems.

### 3-2. NUCLEIC ACID STAINING AND TESTS

Although the major features of morphology are distinct in living cells and in fixed uncolored cells, the bulk of cytological researches have been

made with the advantage of artificial contrast which is introduced by staining or developing a metallic precipitate in cell components.<sup>3</sup> One of the oldest of these techniques is basic staining, which involves the use of dye salts which, upon dissociation, carry the color, the chromophore, in the cation and which therefore form colored salts with the anions of strongly acid substances within the cell (Fig. 6-2C). It was early recognized (e.g., Mathews, 1898) that, if basic dyes were applied in acid solution (after appropriate fixation), this property of cell substances, which is included in the general term "basophilia" of tissues, constituted in effect a test for strongly acidic substances. Specifically, in animal cells these include the relatively uncommon sulfuric acid esters of polysaccharides and the phosphoproteins plus the universal cell constituents nucleic acids, which are orthophosphoric esters of nucleosides. There were many early cytochemical researches based on the supposition that basophilia indicated the intracellular distribution of nucleic acid. One outstanding example is the so-called "chromidial hypothesis," which as applied to metazoan cells held that the basophilia of cytoplasmic structures was evidence of their origin from the nuclear chromatin (see Wilson, 1925, pp. 700ff.). However, most cytologists used basic staining so as to achieve maximum contrast for morphological studies (e.g., iron hematoxylin, applied to material which had been fixed in reagents containing chromic acid). Such technique departed widely from the strict criteria laid down by Mathews for specific staining by salt formation between basic dyes and nucleic acids, and undoubtedly the increased contrast was to a large extent due to adsorption of dye rather than chemical staining (Pollister, 1952a). This distinction was rarely appreciated, however, either by cytologists themselves or by others interested in the chemistry of the cell, and as a result there developed a widespread distrust of attaching any chemical significance whatsoever to the basophilic reaction. This was not dissipated even when van Herwerden (1913, 1914) developed the nuclease technique for identification of intracellular nucleic acid basophilia. The modern use of basophilia for localization of nucleic acids (Mazia and Jaeger, 1939; Brachet, 1942; Pollister, 1950; Kaufmann *et al.*, 1951) stems directly from van Herwerden's work but rests on a much firmer biochemical basis since it is now known that:

1. There are actually two nucleic acids, the pentose type—ribose (RNA), plasmonucleic acid—found in the cytoplasm, nucleolus, and to some extent in chromatin, and the deoxypentose type—deoxyribose (DNA), chromonucleic acid—which is normally restricted to chromatin of the nucleus (Davidson, 1950).

<sup>3</sup> The development of phase contrast microscopy (see Fig. 6-2A and Bennett *et al.*, 1951) has nearly freed cytologists from the necessity of introducing artificial contrast by these methods.



2. There are specific enzymes, ribonuclease and deoxyribonuclease, which act to degrade each type of nucleic acid.

3. The total nucleic acid content may be removed specifically by chemical agents such as trichloroacetic acid.

Thus, sites of nucleic acid are readily identifiable as parts of the cell with basophilia which is removable by acid extraction, and the type of nuclease susceptibility shows which of the two nucleic acids is present (cf. Figs. 6-2C, *F* and 5a, *b*).

Such specific nucleic acid basophilia adds to cell morphology an important chemical datum, showing that, in a cell such as that of Fig. 6-1, for example, there is undoubtedly considerable nucleic acid in the basal zone and nucleolus as well as in the chromatin. More precisely, from such a cytological preparation as Fig. 6-2C it can be concluded that, in these parts of the cell, the nucleic acid concentration is so high that the dye bound as dye nucleate is in high enough concentration to appear as strong visible color in structures no more than  $5\ \mu$  thick (p. 209). To what extent does this approach a complete picture of the distribution of major polynucleotide concentrations within the cell? It must be emphasized that basic staining does not lead to localization of nucleic acid by any of its natural physical properties in the same manner as the natural green color indicates the sites of chlorophyll. Instead, visualization by basophilia depends on the capacity of nucleic acid to bind the cations of basic dyes, which may be mainly through displacement of protein from its natural combination with the residual phosphoric acid valencies of the polynucleotide. This staining reaction may therefore be very complex, and the relation between color and amount of substrate may by no means necessarily be a simple one. From basophilia alone it is impossible to answer such questions as: How strict is the proportionality between basophilia and nucleic acid concentration? Is this proportionality constant or highly variable? What interpretations may be assigned to negative basophilia? Can there be considerable accumulations of polynucleotide which are unaccompanied by any basophilia? Is an increase or decrease of basophilia due to change in amount of nucleic acid or to change in the number of phosphoric acid valences which are available for dye binding? The transition from a cytological to a cytochemical viewpoint poses all such questions and immediately reveals the danger inherent in uncontrolled cytochemical use of staining reactions. Only by an independent method which measures nucleic acid directly can these questions be answered, and in natural ultraviolet absorption of nucleic acid such a method is available (see Sect. 3-4). In general, regions of strong nucleic acid ultraviolet absorption have been found to coincide with those of pronounced basophilia (see Figs. 6-2C, *D*), but there are indications that the amount of dye bound in cells for a given amount of pentose polynucleotide is variable (M. H. Flax, unpublished data), and one extreme case has been reported

in which there was nearly negative cytoplasmic basophilia in cells which, by ultraviolet absorption, were shown to contain considerable nucleic acid (Pollister . . . Breakstone, 1951). In view of these difficulties it is perhaps best to regard basophilia as a useful indicator of sites of major nucleic acid concentration, which then becomes a guide to application of more satisfactory qualitative and quantitative methods. In fact, this has been the role of basophilia in development of modern concepts of the intracellular distribution of nucleic acid. Although the methods of identification by the ultraviolet absorption spectrum measure nucleic acid directly and are therefore potentially applicable to situations where polynucleotide basophilia might be misleading, for the most part ultraviolet studies have proceeded along lines which were clearly foreshadowed by old findings of the basophilic reactions of cells. This is strikingly emphasized also by the fact that practically all the fundamental conclusions about intracellular distribution of nucleic acids which the Caspersson school reached by use of ultraviolet absorption techniques and used in elaborating comprehensive theories of cell function (see Caspersson, 1950) were arrived at independently by Brachet and his collaborators (Brachet, 1944) with only nuclease-digestible basophilia as a guide. Indeed, since Brachet and coworkers used nucleases in combination with basophilia, they were able to detect ribonucleic acid in chromatin, while it was necessarily overlooked in the less specific ultraviolet absorption studies.

There are certain applications of basophilia to qualitative cytochemistry which are of special interest because not only do they demonstrate the presence of strongly acidic substances, but also by specific color changes they appear to indicate something of the intramolecular structure of the acidic substrate with which they combine. The best known of these is the so-called "metachromatic" basophilia (metachromasia) by which certain cellular structures stain red with dyes which appear blue in solution (e.g., toluidine blue and azure). This method was empirically recognized long ago (Ehrlich, 1877; Hoyer, 1890). It has been the subject of a number of chemical and spectrophotometric studies (e.g., Kelley and Miller, 1935a, b; Lison, 1935; Bank and Bungenberg de Jong, 1939; Wislocki *et al.*, 1947; Michaelis and Granick, 1945). Spectrophotometric analysis shows that, whenever these dyes are in water solution, three states are in equilibrium. An  $\alpha$  absorption peak in the red part of the spectrum represents unaggregated dye in the "monomeric" state; a  $\beta$  absorption peak (green) is believed to represent dye in the two-molecule aggregate, or "dimer," state. A  $\mu$  absorption peak (in the blue-green) supposedly represents higher states of aggregation than the dimer which may for convenience be called a "polymer" state. The aggregation, as would be expected, is dependent on concentration, with the result that these basic dyes notably fail to follow Beer's law. In stained cells, basic dyes are, of course, removed from solution and combined with the solid



coagula or precipitates of the substrates. This suggests that the substrates which are colored red have bound the dye in such steric relation that the dimer and polymer association occur, while those colored blue have the dye molecules more widely separated so that substantially all the color is due to the blue monomer. According to this interpretation, the color of the dye is in effect a reflection of the intramolecular structure of the substrate. The polysaccharide sulfuric esters (e.g., chondroitin sulfate of connective tissue) are decidedly metachromatic. A careful spectrophotometric analysis has shown that a metachromasia distinguishes RNA from DNA, a difference which presumably is related to the highly branched structure of the former (Flax and Himes, 1950, 1952).

Methyl green basophilia is another staining reaction which appears to reflect the intramolecular configuration of the substrate. This dye stains normal DNA; with rare exceptions it does not stain RNA. *In vitro* the formation of the salt methyl green–deoxyribonucleate is dependent on the nucleic acid being in a state which forms highly viscous solutions (Kurnick, 1947, 1949; Kurnick and Mirsky, 1949), and therefore reduction or loss of methyl green basophilia of nuclei has been interpreted as evidence of a physical change in the DNA molecule which is similar to that which is accompanied by loss of viscosity of solutions of the acid, a change which is usually called “depolymerization” (Pollister and Leuchtenberger, 1949; Leuchtenberger, 1950; Leuchtenberger *et al.*, 1949; Harrington and Koza, 1951). Such changes in methyl green basophilia of nuclei have been noted to result from experimental treatment (heat, deoxyribonuclease digestion, ionizing radiation) and also to accompany pathological nuclear degeneration.

**3-2a. Nucleal Reaction.** Goldschmidt (1904) and the other adherents of the views embodied in what was called the “chromidial hypothesis” believed that they had in basophilia a sort of qualitative test for chromatin by which they could detect this substance even after its extrusion from the nuclei into the cytoplasm. This was an over-optimistic point of view and led to widespread distrust of cytochemical conclusions from staining results. Feulgen and Rossenbeck (1924) developed a specific cytochemical test for chromatin (Fig. 6-2F) which not only finally disposed of the chromidial hypothesis, *sensu strictu*, but also led to great strides in clarification of the whole problem of intracellular distribution of nucleic acids. For nearly thirty years two different nucleic acids had been recognized by chemists. One, obtainable in quantity from yeast and often called yeast nucleic acid, had been shown to contain a pentose; the other, identical with the acidic component of Miescher’s nuclein but usually later obtained from the thymus gland, contained a sugar that was clearly not a pentose and was generally considered to be sort of hexose (see Levene and Bass, 1931; Davidson, 1950). Feulgen and Rossenbeck discovered that mild acid hydrolysis, which was known to split off the

purine bases from nucleic acid, changed the thymus nucleic acid so that it gave a positive Schiff reaction for aldehydes—a restoration of color to a reduced leukofuchsin, and they demonstrated that this reaction could be carried out not only *in vitro* but also on tissue sections in which it colored brilliantly the chromatin of the cell nuclei. For this reason they called it the “nuclear reaction.” Cytologists were quick to try this new technique, and in a few years the reaction had been demonstrated to be positive on the tissues of a wide variety of animals and plants (Milovidov, 1936). This is an ideal qualitative chemical reaction, highly specific (Fig. 6-2F) for the unique substance of chromatin which Levene *et al.* (1930) eventually showed to be deoxypentonic acid (DNA), not a hexose polynucleotide. The intense color (Table 6-2) is one of its important characteristics. The Feulgen reaction at once demonstrated that the major basophilic component of chromatin and chromosomes of both plants and animals is always DNA and that the cytoplasm never contains this in detectable amount. This was eventually fully confirmed by the analysis of isolated nuclei and cytoplasm (Feulgen *et al.*, 1937; Hogeboom *et al.*, 1948). It thus became quite clear that, whatever the nature of the basophilic substance in the cytoplasm, it was certainly not the same as the nucleic acid of Miescher and Altmann. When, therefore, as a result of the earlier discoveries of pentoses and purine bases in the cytoplasm of developing eggs, Brachet (1942) was led to reintroduce on a wide scale van Herwerden's (1914) long-forgotten nuclease technique and conclude therefrom that the Feulgen-negative basophilic substance of the cytoplasm was a pentose nucleic acid, it was not seriously questioned; likewise, no objections were raised when Caspersson and Schultz (1939) stressed the fact that the ultraviolet absorption spectrum of these same basophilic areas must be due to pentose nucleic acid because these parts of the cell were Feulgen negative. Like basophilia, the Feulgen reaction is not directly dependent on a physical property of nucleic acid but on a chemical reaction which the acid can give after removal of the purines from a part of the nucleotides. (From the results of Stacey *et al.*, 1946, it appears that the deoxypentose undergoes considerable intramolecular change to become a substance which is capable of recolorizing the Schiff reagent.) It must be understood that the very useful specificity, which rarely has been seriously questioned, resides in the release of reactive groups as a result of the hydrolytic process, and it is customary to stain simultaneously a control slide which has not been hydrolyzed.

### 3-3. PROTEIN STAINING AND TESTS

Although the bulk of the solid matter of the cell is protein, the prospect for fruitful cytochemical protein analysis is by no means as bright as for nucleic acids. It is certain that this protein mass must be a very heterogeneous mixture, including countless intracellular enzymes and other pro-



teins of special function. Most of these may never be highly concentrated in any one part of the cell. Even if such an accumulation did occur, the correlation of specific function with readily accessible aspects of the chemistry of the protein molecule is rarely so definite as to offer hope of localizing many specific proteins by techniques similar to those which have just been described for nucleic acids. Natural color, as in hemoglobin, of course, offers one opportunity for a microscopic approach (Thorell, 1947). For the most part, however, methods of microscopic analysis of proteins cannot be expected to give more than information concerning the approximate total amount of the protein mass, the fractionation of which on a microscopic slide is possible to but a very limited extent. For example, a considerable proportion of the histone of chromatin is split off readily (Pollister and Ris, 1947). Nevertheless, the approximate analysis of total protein is information of considerable importance to the broad question of protein synthesis as the prime chemical achievement in growth, cell division, and secretory activity (Caspersson, 1950; Pollister, 1954).

Most methods for protein are not nearly so sensitive as are basophilia and the Feulgen reaction for nucleic acids since the special reactions are almost entirely those of groups at the omega ends of the amino acid residues, and in most proteins (protamine being one exception) no specific reacting group makes up more than a small fraction of the total number of amino acid residues. The reactive groups which have been used cytologically are (a) the dibasic amino acids arginine (Serra, 1944; Thomas, 1946) and histidine (acidophilia, p. 219); (b) the dicarboxylic amino acid glutamic acid (by alkaline basophilia, Dempsey and Singer, 1946); (c) the sulfur-containing amino acids cystine and cysteine (see Lison, 1936; Bennett, 1948); and (d) the aromatic amino acids tyrosine, tryptophane, and phenylalanine (see p. 222).

Specific reactions for proteins in cytological material are all adaptations of well-known spot tests. One of the oldest of these is the Millon reaction for tyrosine and tryptophane (Fig. 6-2B), which was used by Leitgeb to identify the nature of crystals in plant cells as early as 1888. No Millon test is impressive under the microscope, partly because the protein cannot possibly be concentrated enough to give a strong visible reaction on a slide and partly because, since everything is colored, the observer does not have the benefit of the contrast to which he is accustomed in a stained preparation. The test material, if present, is, of course, readily detectable microscopically by objective photometric measurements. The sensitivity of the Millon reaction in visible light is low (Table 6-2). At the visible absorption peak (490  $m\mu$ ), for a protein assumed to give a Millon reaction equivalent to 6.25 per cent tyrosine, the  $E_{490}$  is 0.007, and the protein would have to reach a concentration of over 40 per cent to give a detectable extinction, 0.030, in a thickness of 1  $\mu$ . (At the natural ultraviolet absorption peak, 275  $m\mu$ , the absorption is no more intense.)

The sensitivity of the Millon reaction is nearly doubled if it is measured at 365 m $\mu$ , near the peak in the near ultraviolet (Table 6-2 and Fig. 6-13).

By contrast with the specific spot tests, the widely used method of contrast by acid dyes (in which the color is carried in the anion) gives readily detectable color in parts of the cell where protein is concentrated (Table 6-2). For this reason, a dye of color roughly complementary to the basic dye for polynucleotide is often used in preparing microscopic slides for histological or pathological examination (e.g., basic methyl green and acid fuchsin or basic hematoxylin and acid eosin). It has long been known that this acid dye staining probably has a sound chemical basis (Mathews, 1898), that it is essentially the binding of the dye anions by the cationic groups ( $\text{NH}_3^+$ ) of the diamino acids of the protein to form a salt (for example, what we may call, for convenience, protein fuchsinate with acid fuchsin). There is evidence for the chemical basis of this staining reaction in demonstrations that *in vitro* protein binds acid dyes stoichiometrically (Chapman *et al.*, 1927; Fraenkel-Conrat and Cooper, 1944). The specificity of acidophilia for the amino groups of protein has been demonstrated by Monné and Slautterback (1951), who showed that deamination removes the acidophilia. As with basophilia the staining must be carried out in acid solution (pH 1.5–2.0) so as to preclude binding of the dye in other than salt formation (Mathews, 1898; Leuchtenberger and Schrader, 1950). The basic amino acids constitute so large a proportion of the composition of nearly every protein that acidophilia should be a more sensitive test for proteins than the Feulgen reaction is for DNA (e.g., fast green in Table 6-2). Since protein acidophilia is a measure of diamino acids, it can be employed, in conjunction with either the Millon reaction or ultraviolet absorption, to localize proteins of basic character (Leuchtenberger and Schrader, 1950).

All the protein methods just discussed determine the presence of protein only indirectly, through using one of its side groups to develop color in a reagent, or to bind a dye. Hence, any semiquantitative conclusions drawn therefrom are subject to all the possible errors which have been discussed earlier (p. 214). Protein can be measured directly by the natural ultraviolet absorption spectrum of its aromatic amino acids (Chap. 5, this volume, and Fig. 6-3). The microscopic technique for this has been developed by Caspersson (1940a, 1950), and, as usually carried out, the data obtained are absorption curves of masses of nucleoprotein. The qualitative and semiquantitative ultraviolet methods for these two major cell components, nucleic acid and protein, are therefore discussed together in the next section.

### 3-4. ULTRAVIOLET ABSORPTION OF NUCLEIC ACIDS AND PROTEINS

While chromatin is quite colorless, in the ultraviolet spectrum—at a wave length a little shorter than the region transmitted by glass—it can



be seen that it absorbs heavily, as first shown by Köhler (1904). Thus, if the cell is studied visually on the fluorescent screen or photographed by ultraviolet light, the nucleus is dark and is often fully as sharply contrasted with the lighter background as it would be if the chromatin had been stained with a basic dye (Fig. 6-2D). Of the many observers who were impressed with the striking contrast shown by chromatin under ultraviolet examination, none seems to have realized that this was due to a physical property of nucleic acid until Caspersson published his thesis on the chemical composition of structures of the cell nucleus (1936). In

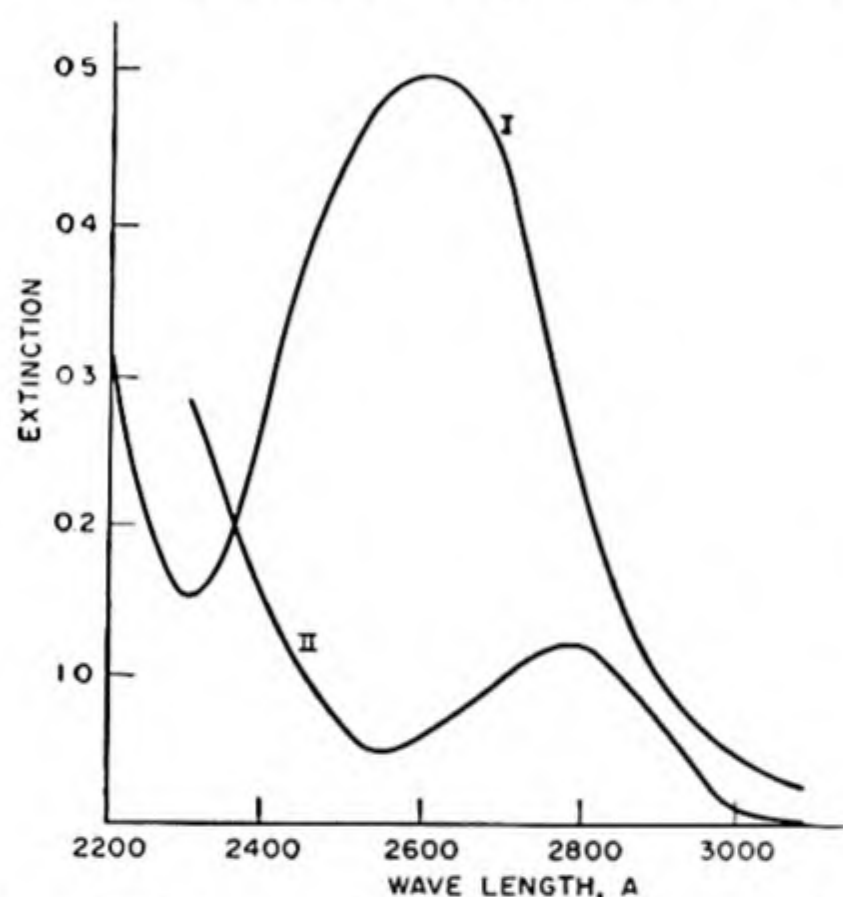


FIG. 6-3. Ultraviolet absorption curve of a 0.02 per cent deoxyribonucleic acid solution (I) compared with that of a 0.2 per cent solution of serum albumen (II). Curves were measured with a Beckman spectrophotometer in a cuvette 1 mm thick. (After Thorell, 1947.)

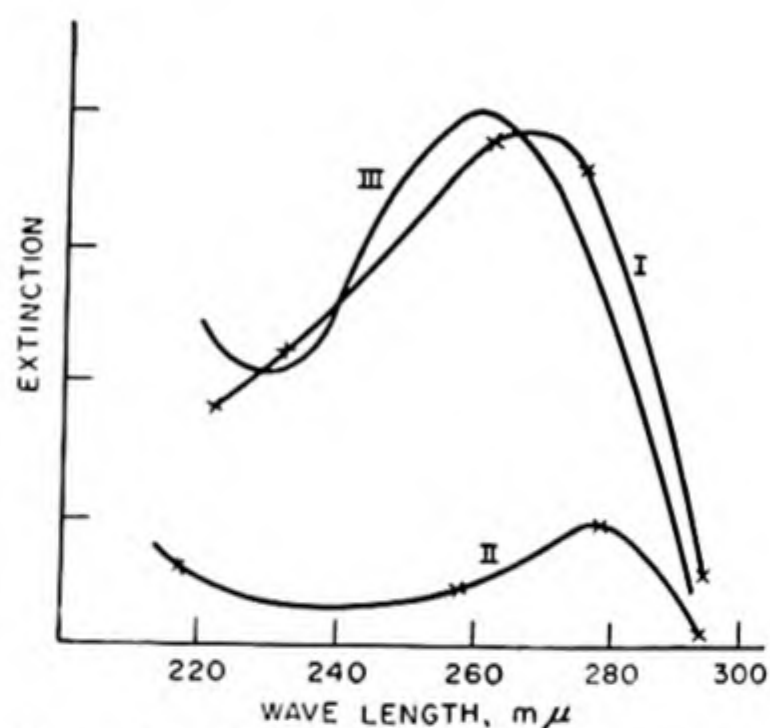


FIG. 6-4. Ultraviolet absorption curves of the salivary gland of *Drosophila*. Curve I is through a chromosome band; II is through an adjacent part of the cell outside the chromosome; III is computed for a 10 per cent solution of nucleic acid. (After Caspersson, 1936.)

this landmark in cytochemistry, Caspersson pointed out that nucleic acid has so strong a natural specific ultraviolet absorption that it can account for the great contrast of ultraviolet pictures (Chap. 5, this volume; Table 6-3 and Fig. 6-3); he showed that the absorption curve through a single chromosome (Fig. 6-4) closely resembles that of nucleic acid, not protein; and he confirmed the opinion that protein could account for but little of the absorption, by digesting the protein with little effect upon the ultraviolet contrast. Numerous subsequent publications of Caspersson and his coworkers have made it amply clear that regions of strong ultraviolet contrast in cells are, as a rule, sites of high nucleic acid concentration (as seen in Figs. 6-2C, D). However, it is a mistake to suppose that such ultraviolet contrast is necessarily an accurate reflection of the intracellular nucleic acid distribution, for in each region the density

shows only the *total* light loss. In ultraviolet light of wave length near that of nucleic acid absorption a large part of the light loss, particularly in fixed preparations, may be of nonspecific character mainly as a result

TABLE 6-3. BEER'S LAW FOR NUCLEIC ACID  
(After Thorell, 1947.)

Deoxyribonucleic acid				Ribonucleic acid			
Concentration, %	Layer thickness, mm	$E$	$k$	Concentration, %	Layer thickness, mm	$E$	$k$
2	0.01	0.373	1.86	2.5	0.01	0.550	2.20
0.01	3.17	0.600	1.89	0.005	3.17	0.340	2.15

The extinction ( $E$ ) and absorption constant ( $k$ ) of RNA and DNA at concentrations of 0.005–2.5 per cent. Preparation according to Hammarsten (1924).

TABLE 6-4. ULTRAVIOLET ABSORPTION OF MAIZE NUCLEOLI  
(After Pollister and Leuchtenberger, 1949b.)

Experiment No.	Number measured	Nucleotides removed by	$E_{254}$	Percentage reduction of extinction
1428E-3	30	.....	$0.690 \pm 0.011$	50.0
1428E-3	30	Hot TCA	$0.347 \pm 0.011$	
1428E-7	23	.....	$0.847 \pm 0.010$	53.4
1428E-7	10	Hot TCA	$0.407 \pm 0.009$	
1428E-5	31	.....	$1.000 \pm 0.014$	52.8
1428E-5	31	Cold TCA	$0.860 \pm 0.011$	
1428E-5	31	Hot TCA	$0.472 \pm 0.008$	
1428E-6	17	.....	$0.843 \pm 0.016$	52.8
1428E-6		Ribonuclease	$0.395 \pm 0.010$	

The reproducibility of the effect of the enzymatic and chemical (trichloroacetic acid, TCA) removal of nucleic acid upon the extinction ( $E_{254}$ ) of whole nucleoli of pollen mother cells of *Zea mays*. (In experiment 1428E-3 the extinction is lower because a very large central cylinder was measured; in 1428E-5 the higher extinction results from using a smaller cylinder.) In each experiment the percentage reduction of extinction indicates the proportion of the light loss (specific and nonspecific) due to ribose polynucleotide. The residual light loss (46–50 per cent) is largely nonspecific, owing to high protein concentration.

of scattering by the dense protein mass (see Table 6-4 and Fig. 6-5b). Specific nucleic acid absorption may be recognized from the shape of the absorption curve (Caspersson, 1950, and Fig. 6-3). Also, the specific polynucleotide absorption can readily be dissociated from the absorption



and scattering of protein by making two photographs or absorption measurements of the same cell, one before and the other after enzymatic or chemical removal of the nucleic acid (see p. 231, Table 6-4, and Fig. 6-5*a, b*). Since the difference between the two is directly dependent upon the natural absorption of the purine and pyrimidine components of the nucleic acid, the latter technique provides an easy and sure method of obtaining evidence of intracellular nucleic acid distribution. The sensitivity is comparable with that of the Feulgen reaction (Tables 6-1, 2). In the specificity of the nucleases lies the possibility of overcoming the

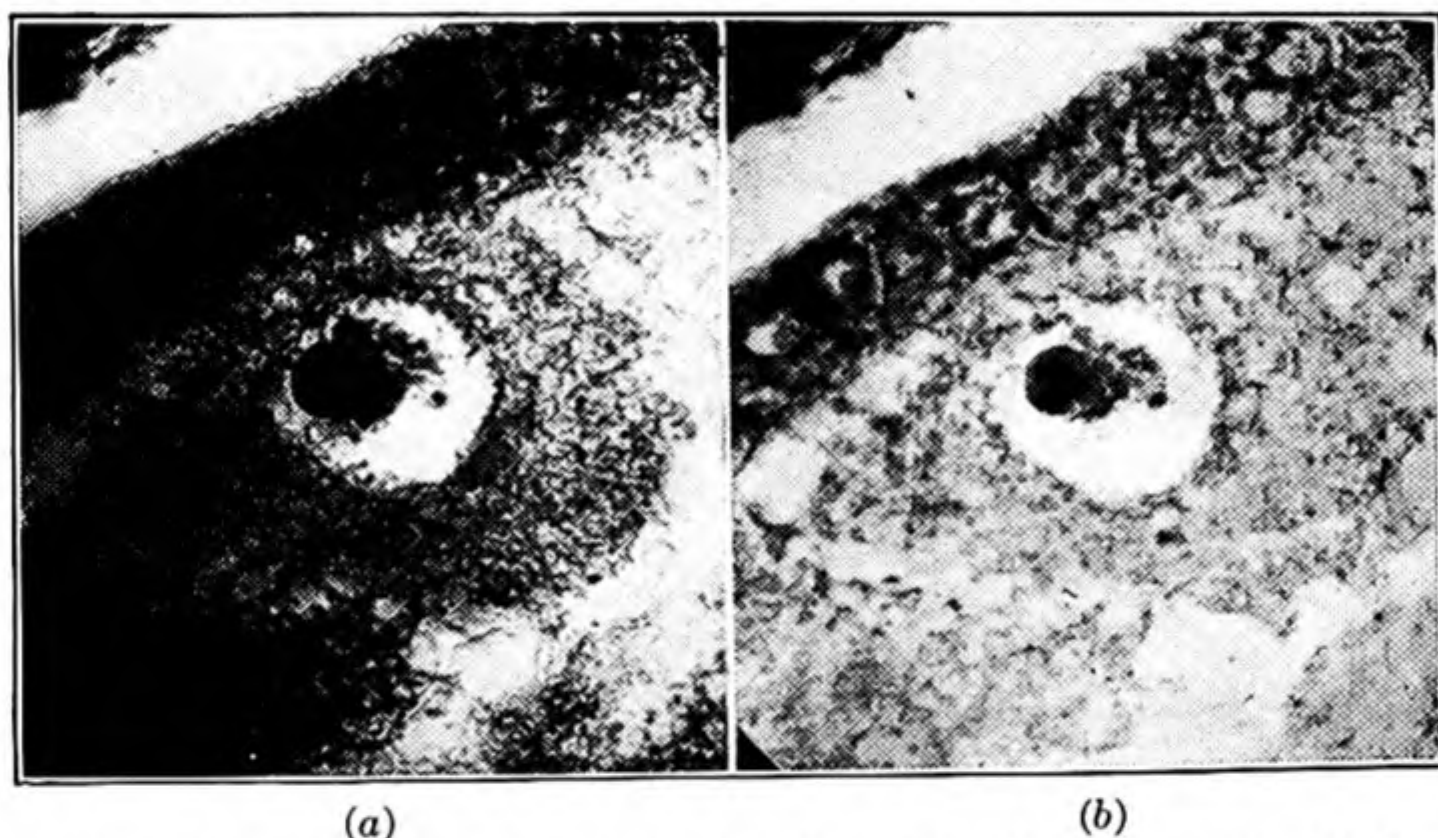


FIG. 6-5. *Test*, left, and *blank* for nucleic acid determination by ultraviolet absorption. Photographs, at  $254\text{ m}\mu$ , of a maize pollen mother cell (No. 1428E-3-54) taken before (a) and after (b) the section had been subjected to hot 5 per cent trichloroacetic acid to extract all polynucleotide. The change in density of the spherical nucleolus is marked. By direct measurement of this nucleolus (Pollister and Leuchtenberger, 1949b) it was found that the extinction of a central cylinder through *a* was 0.750, that through *b* was 0.405, the difference being 0.345. These three values are assumed to represent, respectively: (1) total specific and nonspecific light loss in the part of the nucleolus measured; (2) light loss due to protein, mainly nonspecific; and (3) the light loss due to polynucleotide, mainly specific absorption.

major disadvantage that ultraviolet absorption alone does not discriminate between RNA and DNA (Davidson, 1947; Pollister, 1950).

None of the ultraviolet absorption curves of cell structures is exactly like the curve of pure nucleic acid; there is always distortion, certainly due in part to the associated protein, which characteristically exhibits specific absorption in the region of  $2750\text{ \AA}$  owing to its content of aromatic amino acids (Chap. 5, this volume). Since these constitute but a small percentage of the total amino acid content, the specific absorption of proteins is very low in comparison with that of nucleic acid (Table 6-1 and Fig. 6-3), and, within the region of nucleoprotein absorption which has been most studied ( $2500\text{--}2800\text{ \AA}$ ), protein must be present in 20–50

times the concentration of the nucleic acid to cause an equivalent ultra-violet light loss. The distortion of the nucleic acid curve means therefore that in the cell the nucleic acid is always accompanied by at least several times as much protein.

The intracellular nucleoprotein curves published by the Caspersson group are of two distinctly different types (I and II, Fig. 6-6). In type I the nucleic acid peak is broadened, and the whole right shoulder is shifted toward the longer wave lengths. This shape is not unexpected for nucleoprotein; it seems to be simply the summation of a nucleic acid curve and that of a common protein type, like serum albumen (Fig. 6-3). On this basis, cell regions showing this type absorption have been interpreted as sites where nucleic acid and a typical acid protein ("globulin type") occur together. The type II curves are very different and puzzling; there is less broadening of the nucleic acid peak, and within the long-wave-length slope a second peak is indicated by a distinct shoulder. For a variety of reasons (see Caspersson and Thorell, 1941), curve II has been held to localize nucleic acid accompanied by markedly basic protein, called "histone type" or "diamino-acid-rich" protein (Caspersson, 1940a, 1950). The strongest evidence for this interpretation of the type II curves was that certain nucleohistone preparations showed a protein peak apparently shifted toward 2900 Å. However, when histones quite free of nucleic acid were finally obtained, it was found that they did not show such a shift of absorption, the peak being near 2750 Å as in typical proteins (Mirsky and Pollister, 1943, 1946). There remains therefore no certain explanation of the peculiar shape of the type II curves.

The nucleoprotein curves have been the basis for detailed speculations concerning the roles of basic and acid proteins in cellular physiology (Caspersson, 1950). For the general cytologist, perhaps the greatest significance of these curves is that they emphasize unmistakably what

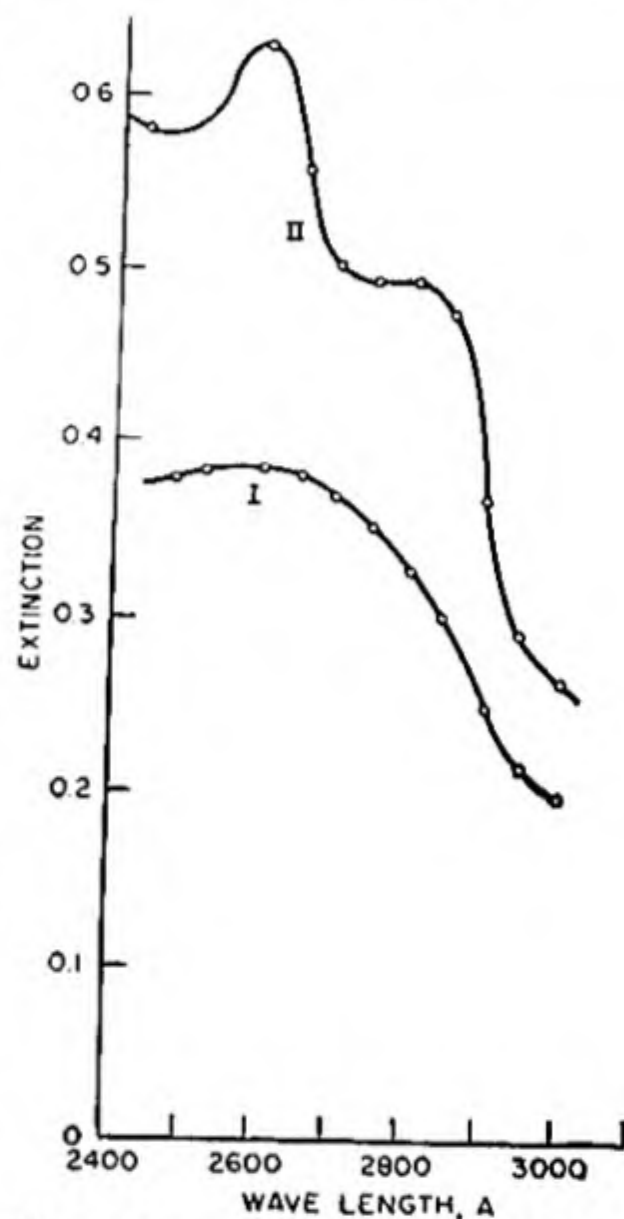


FIG. 6-6. Absorption curves of the cytoplasm of cells of differentiated (I) and undifferentiated (II) renal tubules of the chick embryo. Curves of the I type are considered to represent ribonucleic acid with protein of the "globulin type"; the type II curves are believed to indicate the presence of ribonucleic acid accompanied by considerable basic protein, "histone type." (After Caspersson and Thorell, 1941.)



might otherwise be overlooked, namely, that, however striking the basophilia and ultraviolet absorption of nucleotide-rich parts of the cell may be, this by no means signifies that they are strongly acidic pools of nearly pure nucleic acid. Instead, the absorption curves clearly show that protein is usually present in much greater quantity than the nucleic acid. This situation must always be recalled in any attempt to interpret chemically the results of staining reactions, and it must be the starting point for all speculations concerning the role of nucleoproteins in intracellular physiology (see Pollister, 1952b).

#### 4. QUANTITATIVE MICROSCOPICAL METHODS

##### 4-1. VISUAL COMPARISON

The qualitative cytological methods which have just been discussed lead to localization of a substance within a cell by its absorption, "contrast" in the language of a microscopist, which is detectable visually. Cytologists often speak of the intensity of a stain or color reaction as weak, strong, very dark, etc. Of course these terms imply semiquantitative evaluation of the concentration of the component which is responsible for the color. When two similar objects are side by side in an evenly illuminated microscopic field, or in the two half fields of a comparison eyepiece, visual matching appears to be as accurate as objective photometric measurements. For example, with the comparison eyepiece, Bauer (1932) arranged a series of slides in order of intensity and was thus able to work out the relation of intensity of the Feulgen reaction to time of hydrolysis, which was essentially like that later worked out by Di Stefano (1949) from photoelectric measurements. For objects of the same size proper visual comparison is, then, a rough indication of relative amounts—if two objects are equally dark they may be assumed to have approximately the same amount, and if they are different, the darker one may be said to contain more reacting substance. The same conclusions regarding relative concentrations may be drawn of two bodies of equal vertical thickness (equal absorbing path). More often, the quantitative question which faces a cytologist cannot be answered, even roughly, by visual comparison. For example, one often wishes to know relative amounts in two objects of very different size. It is uncertain to what extent by visual study a microscopist can determine whether two such unequal objects have the same intensity (a rare condition probably); for the relative sizes of the contrasting surroundings introduce considerable difficulty. If this match could be accurately made, a fairly good estimate of how much more the larger object contained could be computed from the dimensions of the objects.

The examples cited illustrate the range of visual microscopic comparison. If, to mention a very common experience, one cytological

object is *both larger and more lightly colored* than another, the cytologist is almost completely helpless to answer the obvious question of whether the decrease of color is entirely due to dilution in the larger mass. The relation of volume to light absorption is easily computed from an actual figure, a measurement of extinction, but such a quantitative datum is absolutely necessary. No amount of experience can train a cytologist's eye to operate as a microscopic photometric device. These measurements must be made with objective photometers, with which a transmission is measured from which concentration may be estimated and amount computed (where the form and homogeneity of the cytological object are favorable).

#### 4-2. PHOTOMETRIC TECHNIQUE

The simplest photometer is a photographic plate, which can be used to determine relative intensities, from comparison with a density-intensity calibration curve. This curve may be independently measured and used for a whole series of plates (Caspersson, 1936; Pierce and Nachtrieb, 1941) or may be measured from a series of intensities through a rotating sector (Cole and Brackett, 1940) or a calibrated wedge (Uber, 1939) which is photographed on the same plate with the cells. The photographic method seems at first glance easy and obvious, a simple modification of the technique of photomicrography. For accurate results, however, it is far more complicated. Plate exposure and development must be rigidly standardized, and the negative density must be measured with a fairly elaborate photoelectric apparatus. The latter must, in fact, be nearly as sensitive as a photometer for direct measurement of microscopic slides—hence, in most cytological work, the latter is an easier technique. Photographic photometry is indispensable for some problems, for example, where ultraviolet absorption measurements are to be made upon living cells (Thorell, 1947; Malmgren and Hedén, 1947; Mellors *et al.*, 1950).

For direct absorption measurements of fixed preparations at a single wave length, the relatively simple device indicated in Fig. 6-7 is adequate (Pollister and Moses, 1949). Photomultiplier tubes are sensitive enough to allow measurement of areas less than  $1 \mu^2$ , for all methods for nucleic acid and protein. Other devices employing photomultiplier tubes have been described by Lison and Pasteels (1951) and by Pollister (1952c). The data obtained are pairs of measurements, a first ( $I_x$ ) through part of the cell, a second ( $I_0$ ) through an empty part of the slide, outside the section. From these, transmission ( $T$ ) can be computed (as  $I_x/I_0$ ) and extinction either computed (as  $\log_{10} I_0/I_x$ ) or obtained from a conversion table (Brode, 1943).

If absorption curves are to be measured, the apparatus described must be supplemented by means of dispersing the spectrum either before or



after the microscopic preparation. There are many possible types of apparatus for this sort of procedure (see Caspersson, 1950; Loofbourow, 1950; Mellors *et al.*, 1950; Blout *et al.*, 1950). Since in the visible spectrum there are objectives and condensers, corrected both for chromatic and spherical aberrations, the measurement of visible absorption spectra is relatively simple, involving merely movement of the wave-length drum of the monochromator. For ultraviolet absorption measurements, Caspersson (1936) originally used the Zeiss-Köhler apparatus and measured absorption at each wave length by photography. This whole instrument was designed for photography at one wave length, quite

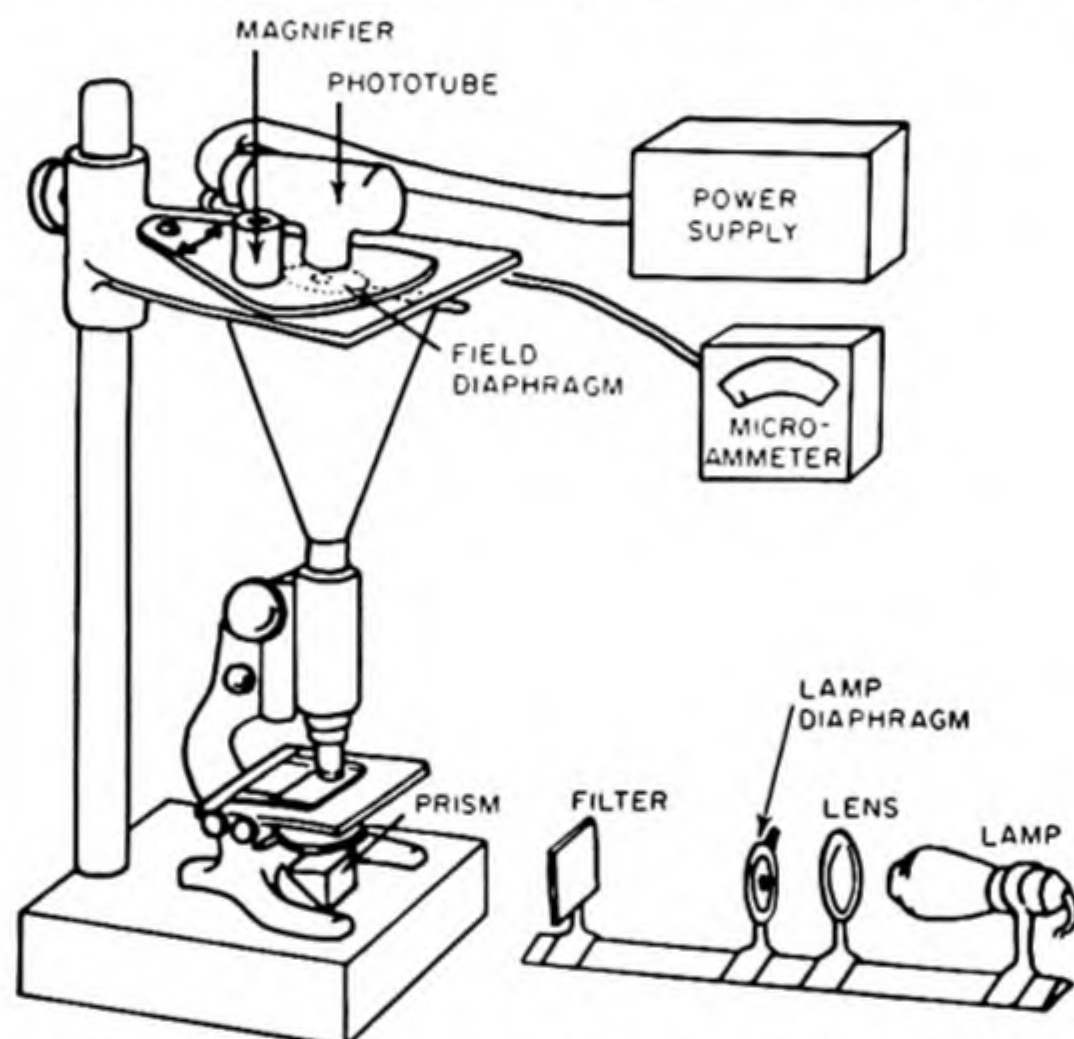


FIG. 6-7. Diagram of a simplified apparatus for microphotometric study of cytological preparations. (After Swift, 1950.)

uncorrected for chromatic aberration, and its use for absorption curve measurement by photography is extremely tedious. For each wave length both condenser and objective must be refocused, and it is necessary to carry out two measurements or make two photographs at each wave length. This uncorrected optical system has been used for all the extensive work of the Caspersson school, with many additions (Fig. 6-8), such as an achromatic grating monochromator, extremely sensitive photoreceivers (measuring currents of the order of  $10^{-14}$  amp) and a polarizing prism and special mechanical stages (Caspersson, 1950). The development of achromatic reflecting objectives and condensers (Fig. 6-9) (Brumberg, 1943; Burch, 1947; Grey, 1950; Norris *et al.*, 1951; Barer, 1951) makes the problem of optical apparatus for ultraviolet absorption measurements essentially as straightforward as in the visible spectrum. With the instrument which has been developed by Sinsheimer a com-

plete absorption curve can be run mechanically, without refocusing; a density-wave length curve is recorded on a drum; and by a beam-splitting mechanism and chopping the beams at two frequencies it is possible to compensate for the transmission of the empty part of the slide so that no second curve is necessary (Loofbourow, 1950). Many details of technique will be found in such references as Caspersson (1936, 1950);

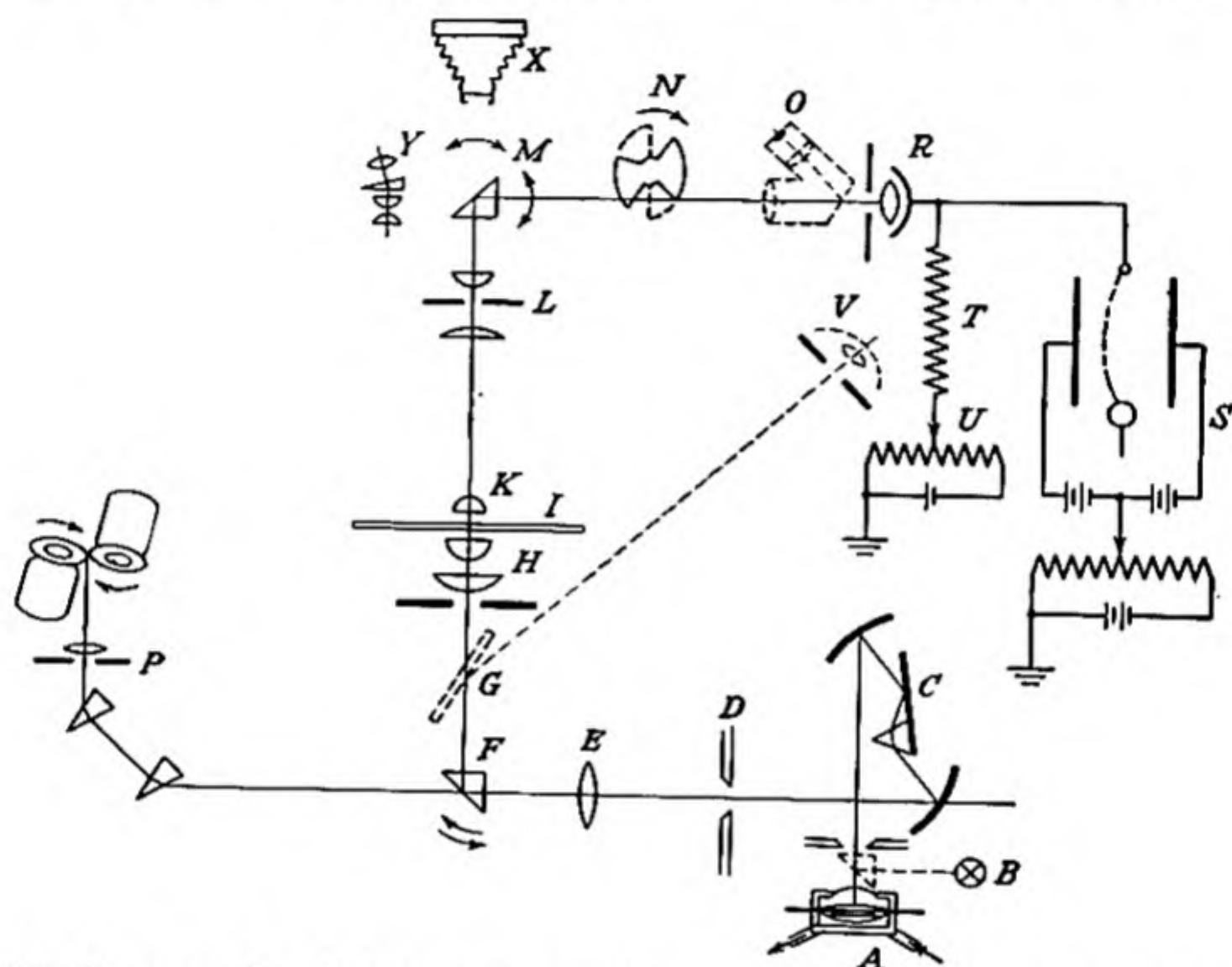


FIG. 6-8. Diagram of main instrument used in measuring ultraviolet absorption with high accuracy and stability. A, mercury lamp; B, tungsten band lamp; C, monochromator; D, exit slit of monochromator; E, lens; F, movable 90° quartz prism; G, quartz plate, used with photocell to compensate for changes in the lamp; H, condenser; I, object on slide; K, objective; L, ocular with adjustable diaphragm; M, accurately movable prism of fused quartz; N, rotating sector; O, telescope for centering; P, Kohler's rotating spark gap arrangement; R, photocell; S, electrometer; T, leakage resistance; U, four-step potentiometer; X, camera; Y, Kohler focuser for the ultraviolet, interchangeable with prism M. (After Caspersson, 1950.)

Gersh and Baker (1943); Thorell (1947); Pollister and Ris (1947); Pollister and Moses (1949); Swift (1950); and Pollister (1952c).

An extreme simplification of the problem of instrumentation for microspectrophotometry is to regard the whole apparatus as merely a somewhat more complicated optical pathway than that in the conventional devices which use absorption cuvettes, and to consider the microscope as no more than an aid to locating an extremely small analytical sample symmetrically in the optical pathway and delimiting the area to be measured. If computations of concentrations and amounts are to be



made, it is obviously necessary to take into account the problems raised by such factors as the angle of the illuminating cone from the condenser, the probable variations of path length, and scatter into or outside the area of measurements as this cone is changed. These questions are con-

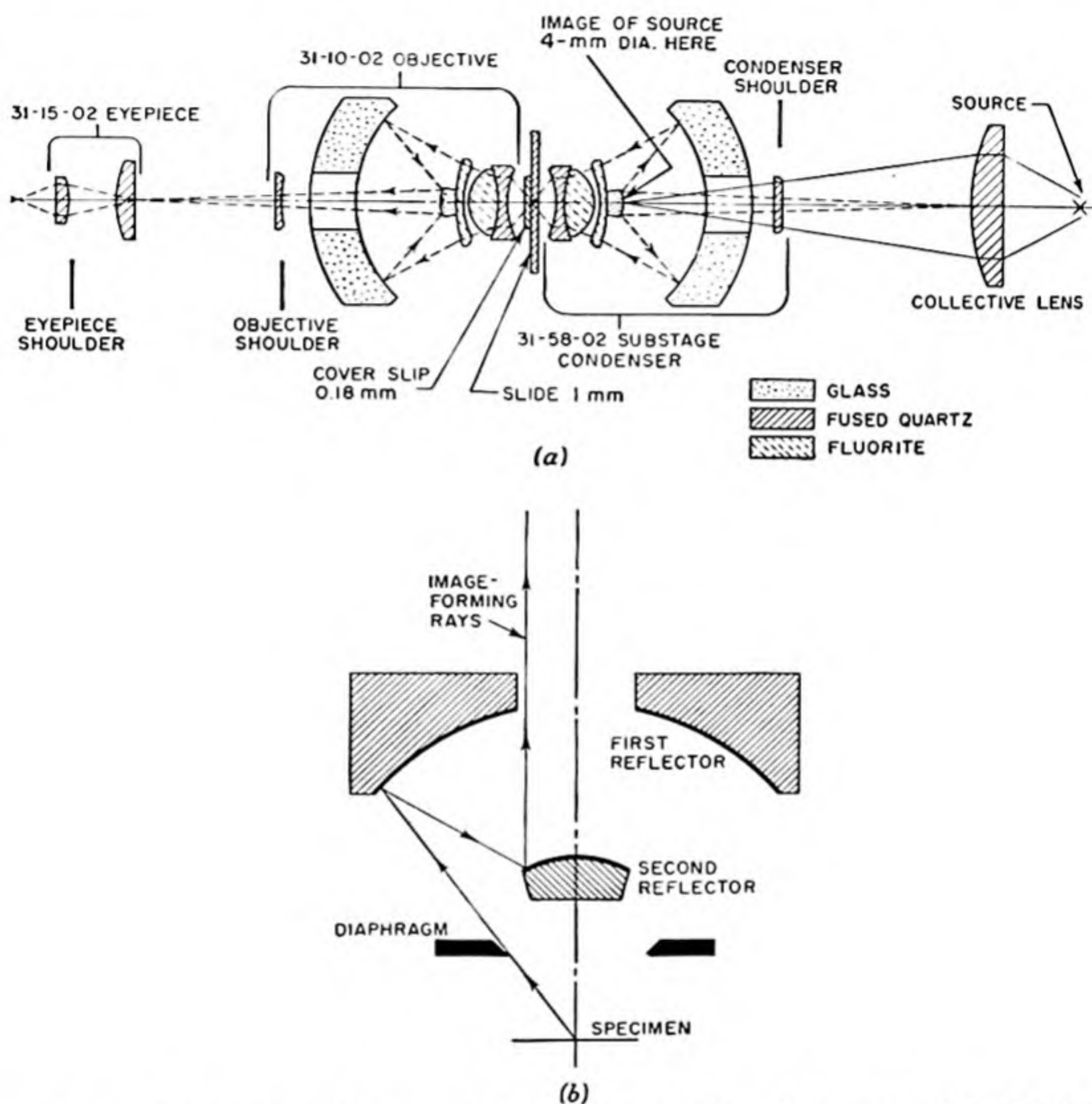


FIG. 6-9. (a) Diagram of the optical components of a microscope with reflecting-refracting condenser and objective, for use in the visible and ultraviolet spectrum. (*The Bausch and Lomb Optical Company.*) (b) Diagram of a totally reflecting objective, a design with chromatic correction over a wide extent of the visible, ultraviolet, and infrared regions of the optical spectrum. (*Courtesy of A. J. Kavanagh and The American Optical Company.*)

sidered at length, from both the theoretical and experimental viewpoints, in such references as Caspersson (1936, 1950); Uber (1939); Thorell (1947); Swift (1950); and Davies and Walker (1953).

Caspersson, especially, has discussed at some length the problems raised by image formation, a treatment which seems to make the whole ques-

tion of microspectrophotometry considerably more difficult than in the simplification suggested. He believes that if the intensity distribution in the microscopic image is to correspond in every detail with that in the cell, a requirement for absorption measurements, then the demands on the microscope are essentially identical with those set forth by Abbé for highest resolution. Others have suggested that an absorption microscope can perhaps be a compromise between the simplified optical system of such instruments as colorimeters and that for the sharpest images at high magnification (Norris *et al.*, 1951; Grey, 1952; Kavanagh, 1952).

#### 4-3. SOME ERRORS OF QUANTITATIVE MICROSPECTROPHOTOMETRY

In practice, an apparatus for microscopic absorption is indeed much like a colorimeter or a spectrophotometer, except for the introduction of a microscope into the optical pathway, and the actual absorption measurement is essentially the same— $I_x$  is intensity measured through the cell while  $I_0$  is a second reading through an empty part of the slide, outside the section. Such quantitative absorption data are easily obtained, but the successful evaluation of the results must take into consideration many possible sources of error which arise from the nature of cytological material and the fact that the microscope is used. Most substances within the cell are in a physical state very different from the dilute solutions measured in a colorimeter or spectrophotometer. The proteins and nucleic acids are very concentrated and if fixed, possibly even when unfixed, are more like solid precipitates or gels than solutions. Very little is actually known about the extent to which this physical state can affect the operation of Beer's law because no extracellular model for such a study is available. Solutions of nucleic acid which approach that which occurs in cells (1–5 per cent) appear to give the same  $k$  value as dilute solutions (Table 6-3), and the absorption curve of such concentrated solutions measured in the microspectrophotometer matches very closely that obtained on dilute solutions (Fig. 6-10). Nevertheless, it is difficult to escape a sus-

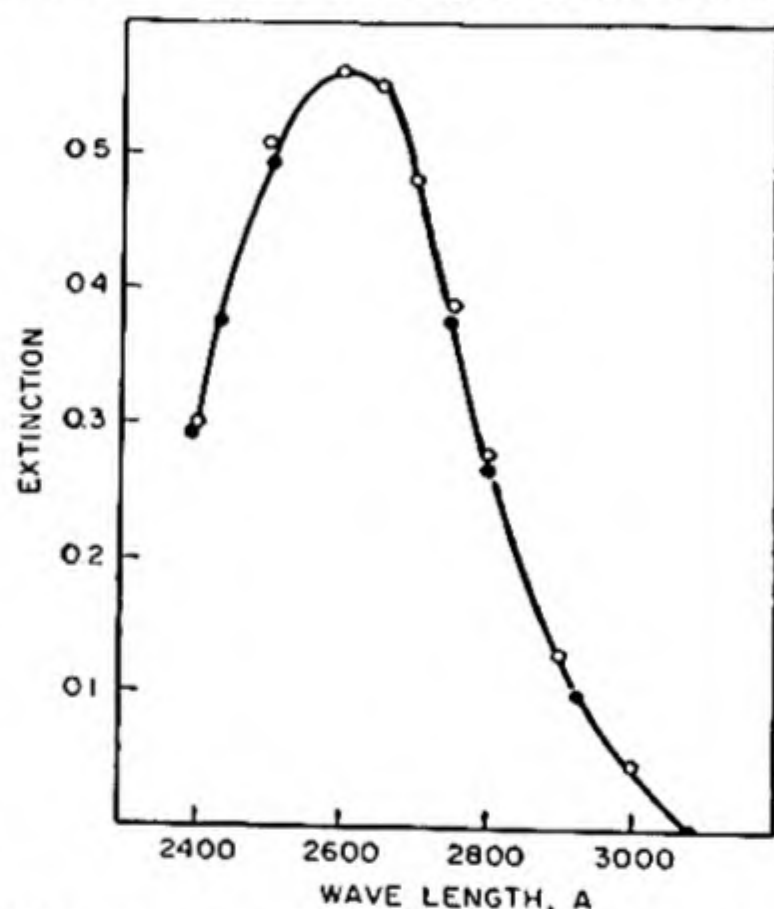


FIG. 6-10. Absorption spectrum of a 2.5 per cent ribonucleic acid solution, obtained (1) in a cellophane bag with a microspectrographical arrangement (solid circles) and (2) in a 10- $\mu$  Scheibe cuvette with a photoelectrical absorption spectrograph according to Warburg-Negelein (open circles). (Redrawn after Thorell, 1947.)



picion that, in the complex nucleoprotein association within the cell, the resonance conditions may be significantly different from those of the isolated nucleic acid and protein in solutions (see Chap. 5, this volume), and hence any computations of absolute concentration or amounts from standards obtained on solutions must be considered provisional. For relative concentrations this is less important, for it is a much less unlikely assumption that  $k$  is constant throughout the limited range under study.

Deviations from Lambert's law that extinction is proportional to thickness are uncommon sources of error if photometric analysis is properly carried out, since there are no conditions within a thick sample which differ from those within a thin one. It has been suggested that, within a cytological preparation, error may arise because the color reaction occurs only at the surface of the section, or because a colored product piles up to form a sort of opaque screen on the surface. This has not yet been found. Conformity to Lambert's law is easily tested in cytological preparations, and it has been repeatedly demonstrated that light loss is proportional to thickness of the absorbing layer (Pollister and Ris, 1947; Pollister and Swift, 1950). A possible source of failure is dichroism as a result of orientation of chromophores (Commoner, 1949; Commoner and Lipkin, 1949). No case of such error has yet been detected in visible and ultraviolet studies (Pollister and Swift, 1950), and there is evidence that moderate nucleic acid orientation would have little effect on the ultraviolet absorption measurements of cells carried out with unpolarized light (Thorell and Ruch, 1951). It is perhaps a safe rule that dichroism is unlikely to be a complicating factor except in objects which are conspicuously birefringent (e.g., skeletal muscle). Marked dichroism is a potential tool for study of molecular orientation within the cell. For example, Caspersson (1940b) has demonstrated that the ultraviolet dichroism of grasshopper sperm heads is due to orientation of the pyrimidine chromophores, and infrared dichroism has been employed to detect orientation of protein polypeptide chains (Goldstein, 1950).

When carrying out *in vitro* photometric analysis, the usual method of isolating specific absorption from nonspecific is by subtracting the light loss of a blank, which is either the solvent alone, or a solution of the sample substance in which a color test, which is the basis of the photometric analysis, has not been developed. The wide usefulness of the photometric approach in quantitative chemical analysis depends largely upon these simple methods of extracting the essential datum from what is actually in most cases an extremely complicated optical phenomenon (see Chaps. 1 and 5, this volume). The cytological use of photometric analysis likewise depends on, in one way or another, the relation of light loss within the cell to specific absorption of a given chemical substance. The elimination of the nonspecific component has been best achieved in methods of photometry of color reactions for nucleic acids and proteins.

which may be measured by visible light (Pollister, 1950, 1952a). It happens that when tissues are so fixed that the cell consists of little more than nucleoprotein, all cellular structures have very nearly the same optical dispersion, and it thus becomes possible to mount the specimen in a medium (an oil) which matches the refractive index at any wave length. Under these conditions unstained structures are invisible even by dark-field or phase contrast, showing that nonspecific light loss is negligible. If such material, colored by a reaction, is measured while mounted in oil at or near the appropriate refractive index, practically all the light loss may be assumed to be due to specific absorption by the chromophore of the test. [One possible source of error is that of anomalous dispersion near the absorption peak of the chromophore as pointed out by Scott (1952) and Ornstein (1952).] Another approach is to measure a cell twice, first before (a blank) then after (a test) development of color, a procedure which has been followed with the Millon reaction for proteins (Pollister and Mirsky, 1946; Pollister, 1950). Another method is that used in photometry of the natural absorption of nucleic acids, in which the blank is the second measurement made after removal of the nucleic acid by nuclease digestion or chemical extraction (Fig. 6-5 and Table 6-4). This is somewhat less satisfactory than the protein blank because the component of the nonspecific light loss due to nucleic acid is also removed and thus becomes added to the apparent specific chromophore absorption.

When the refractive index of the mounting medium is markedly different from that of the section (e.g., when an unstained section is in water), the nonspecific light losses become appreciable. The methods of ultraviolet microspectrophotometry have been applied either to living cells or to sections which are mounted in glycerin, after either freeze-drying or fixation (e.g., in acetic alcohol). In the two former materials nonspecific light loss is believed by Caspersson (1950) to be minimized in some cases by the absence of sharp phase boundaries. In nearly all fixed material the nonspecific light loss is always considerable (Fig. 6-2D and 6-5). Apparently no mounting medium for ultraviolet studies closely matches the refractive index of such fixed sections. Hence ultraviolet absorption studies must always grapple with the problem of estimating the scatter and internal reflections. As Caspersson (1950) has said, "the most important of all conditioning factors for quantitative microspectrography is the elimination of the sources of errors caused by these factors." Caspersson elected to estimate the nonspecific light losses, where appreciable, in the preparations, not by a measured blank as described, but by the unusual method of computing them from analogy with the light losses which he had previously studied in solutions of colorless salts—in which, of course, all light loss was nonspecific. In spite of the urgency and priority of this problem for any quantitative interpretation of the nucleoprotein ultraviolet absorption curves, there has never been a complete



explanation of how the nonspecific light losses can be estimated and subtracted from the compound measured curve of intracellular nucleoprotein. The nonspecific light loss in a cell becomes evident as apparent absorption outside the spectral region of specific absorption, in the case of nucleoproteins above 300 m $\mu$ . When extinction approaches zero near this point, it is clear that one is perhaps justified in the assumption that scattering and reflections cause no light losses in the shorter wave length region of strong specific absorption, although the possibility of anomalous dispersion near the absorption peak can by no means be ignored (Scott, 1952). Often, however, the light loss at the nonspecific zone is from one-third to one-half that at the maximum of the specific absorption. It is not clear why in one such case "the loss of light was assumed to depend equally upon reflection and Rayleigh's light scattering" (Hydén and Hartelius, 1948), while in another instance it was assumed that "at 3100 Å light-dispersion conditions the whole absorption and is inversely proportional to the fourth power of the wave length" (Caspersson and Thorell, 1942). Where there is obviously considerable nonspecific light loss, it seems logical to expect to see, side by side, the uncorrected and corrected curves, but there has never appeared an intracellular nucleoprotein curve from which nonspecific light loss has been subtracted. In some cases a curve of "light-dispersion" has been published with the measured light.

In photometric analysis it is a very uncommon procedure to attempt to account for a substantial amount of the nonspecific light loss by computation, probably because it is often difficult to determine the optical constants to be used even with simple solutions or suspensions. Caspersson has chosen to do this with an immensely complex unknown sample, a nucleoprotein mass in a cell, and a full evaluation of the success of this attempt must await more complete details of the computations in specific cases.

The possibility of a change in light loss or in cell structure as an effect of the radiant energy is not very great in measurement of absorption in the visible spectrum. On the other hand, such an effect is rather to be expected when working in the middle ultraviolet range, since it is a common experience that many substances rapidly lose their specific absorption upon exposure to this higher energy radiation. Caspersson was aware at the very start of his researches (1936) that this might occur, and presented experimental evidence that, *in vitro*, the absorption of nucleic acid was less sensitive to ultraviolet radiation than were free guanine and adenine. Caspersson (1936, p. 22) remarked that, "Die Messung dieses Effects ist im mikroskop technisch ausserordentlich schwer, da im mikroskopischen Präparat Deformationen auftreten." Sections of fixed material mounted in glycerin are extraordinarily stable, and an exposure to intense 254 m $\mu$  radiation for many hours causes no measurable reduc-

tion of extinction or noticeable change of appearance. When living cells are studied, however, the possibility of both types of alteration must always be kept in mind. The physical changes induced in nuclei by radiation, presumably of the sort Caspersson noted in 1936, were later pointed out by Brumberg and Larionow (1946), and considered in much more detail by Ris and Mirsky (1949), who believed that nearly all published ultraviolet photographs of living cells show signs of radiation injury, in that the nuclear details are too conspicuous.

Although there is some room for dispute about the exact nature of the effects of ultraviolet radiation on the structure and absorption of living cells, there is little question but that "the living cell is, as a rule, an unsuitable object for microspectrophotometric studies," because of its great motility and because great structural changes occur during irradiation (Caspersson, 1950, p. 57). This point of view is different from that of earlier years, when great emphasis was placed on the advantages of applying these methods to living cells (e.g., Caspersson, 1947, p. 127: "It must be possible to apply the results directly or indirectly to the living cell itself"). Mellors *et al.* (1950) have shown that these particular obstacles to ultraviolet studies of living cells are by no means insurmountable. Using a microscope with achromatic reflecting optics, they dispersed the light after it had passed through the microscope, and photographed the whole ultraviolet spectrum of a mercury vapor lamp on one negative. With very sensitive emulsions, it was found that up to 85 such photographs could be taken before the cell became injured to the extent that mitotic division could not proceed. There may be many physiological problems which can be profitably attacked directly by this method. For studying the question of the nucleoprotein composition of cells, however, the wide range of specific absorption in living cells seems an unnecessary complication, and "for most problems suitably extracted objects give the cleanest data" (Caspersson, 1950, p. 57).

A major task in making significant absorption studies of cells is that of selecting the region of the cell to be measured. All of the cytologist's experience and special craft are sometimes called upon in overcoming this difficulty. How can the absorption of particular cellular structures be isolated from that of adjacent structures and from a surrounding visibly structureless sort of background? This is accomplished easily enough if a highly specific absorption is localized in a particular structure, as is chlorophyll in chloroplasts or the Feulgen reaction in the nucleus. The successful quantitative application of the latter (Pollister, Swift, and Alfert, 1951) is in large part due to its high specificity and sharp localization (Fig. 6-2*F*). When, on the other hand, a reaction is widespread, often to nearly all parts of the cell, it is difficult to determine the absorption of one particular component. Examples are protein tests (Fig. 6-2*B*), basophilia, and ultraviolet absorption, or cases where the cytoplasm



give a marked plasmal reaction with Schiff's reagent. The structures may be isolated by crushing or smearing the cell, the method used by Caspersson (1936) with components of the salivary gland nucleus and by Ris and Mirsky (1949) with nuclei. This is, however, a procedure by no means always applicable. The problem is probably best solved by sectioning tissue appropriately. Nuclear and nucleolar absorption are thus easily isolated if sections are cut approximately equal to or less than the respective diameters of these cell components. The technique of sectioning material in a much lower range of thickness has been developed for electron microscopy (Hillier and Gettner, 1950); sections can be cut  $1\ \mu$  or less, and thus the absorption of single mitochondria or secretory granules can possibly be studied without interference from cytoplasmic overlay or underlay. Until now mitochondria have been studied only where a considerable number are fused to form a large body, the nebenkern of the insect spermatid (Leuchtenberger and Schrader, 1950). A similar advantageous concentration of small bodies (e.g., microsomes) can be accomplished by ultracentrifuging (Lagerstadt, 1949).

In studies of living cells, this problem of isolating the absorption of a particular component seems nearly insolvable. Rarely, if ever, can it be arranged that the nucleus is relatively free of overlying or underlying cytoplasm; either this must be ignored (Caspersson, 1939; Thorell, 1947; Mellors *et al.*, 1950) or a correction must be attempted by measuring the cytoplasm, estimating the thickness above and below the nucleus, and subtracting the proportional cytoplasmic absorption, assuming that it is homogeneous (Caspersson, 1936). This assumption is likely to be incorrect since in many cells the ultraviolet absorbing material is especially concentrated just outside the nuclear membrane. Much more difficult is any sort of measurement of absorption of a nucleolus, and it seems inadvisable to attach much significance to the apparently uncorrected absorptions of small nucleoli in living cells (Thorell, 1947).

The problem of fixation for chemical cytology is of major importance, even though it has not received much attention, cytochemistry having merely taken over some orthodox cytological techniques. These are by no means all suitable methods of observing tissue for cytochemical study. For example, the best fixation of the complete structural details of cells is accomplished by use of mixtures which include osmium tetroxide (osmic acid,  $\text{Os}_2\text{O}_4$ ) and chromium trioxide (chromic acid,  $\text{CrO}_3$ ), (Fig. 6-2A), but these fluids are of extremely limited application in cytochemistry. The chrom-osmium mixtures always tend to overemphasize phase boundaries, they introduce color into the section, and, after their use, practically no fractions can be removed chemically or enzymatically. Cytochemical fixation, as a rule, has been a compromise between the requirements of accurate preservation of morphology and those of photometric analysis and fractionation (Pollister, 1952a). Two colorless

reagents have been most widely used, acetic acid-alcohol and neutral formalin. The former has the advantages of conserving little but nucleoprotein, and this in a state where fractions are readily removable; but acetic acid-alcohol certainly introduces gross morphological artifacts especially in lipid-containing structures such as mitochondria. Formalin introduces less artifacts, but it does preserve many other substances in addition to nucleoprotein and has a tendency to react vigorously with proteins (French and Edsall, 1945) which may quite possibly lead to considerable confusion in interpretation of staining and tests, or even of ultra-violet absorption.

The selection of the exact spot to be measured in the cell is also a difficult task. If the material is homogeneous over a considerable area, the size of the sample is relatively unimportant. The Caspersson group, as a rule, measures a very narrow cylinder (less than  $1 \mu^2$  in area). This has the merit of being so small that in most cases great variations of absorption across the field are unlikely. It has the disadvantage that the spot may not be representative of any considerable part of the cell. All other workers (e.g., Gersh and Bodian, 1943; Pollister and Ris, 1947; Leuchtenberger, 1950; Swift, 1950; Lison and Pasteels, 1951; Panijel, 1951) have measured the transmission of larger areas, often entire nuclei. Sometimes these larger areas are fairly homogeneous, but more often the absorbing material is to some extent concentrated in scattered masses among which there is a sort of continuum of relatively lower absorption. This heterogeneity introduces into the absorption measurements an obvious unavoidable error (conveniently called the "distributional error") which was first pointed out by Caspersson (1940a). This leads to lower extinction values, never to higher. The error is best understood from considering the actual conditions of measurement of two extreme conditions, one where absorbing material is evenly distributed throughout an area and another where the same amount of absorbing material is concentrated in one-half the area, the remainder being free of absorbing material. Suppose the concentration in the first case is sufficient to cut the light down 90 per cent, i.e., the transmission is 10 per cent and the extinction is 1.0. In the second case the light loss will be as follows: Since the concentration of absorbing material in the dark half is doubled, its extinction becomes 2. This means that this half will transmit 1 per cent of the light which falls upon it; and, since this darker portion is one-half the total area measured, the amount of light passing through this absorbing region to the photoreceiver is 0.5 per cent of the total light. The lighter, nonabsorbing half, however, transmits all light, which is one-half the total amount; hence the reading on the instrument will indicate a transmission of 50.5 per cent. The equivalent extinction is 0.306, less than one-third the true value (1.0) obtained when the material is evenly distributed. This case is an example of extreme heterogeneity which



obviously should never be measured as a whole object. The error in measuring sparsely distributed chromosomes on a metaphase plate would perhaps be comparable with this. As the heterogeneity decreases, and the darker and lighter parts of the object come to contain more nearly like amounts of absorbing material, the distributional error rapidly decreases. For an extensive theoretical treatment of the distributional error see Ornstein (1952) who considers several methods of minimizing or correcting for the error.

There is an error involved in the summation of transmissions where intensity distribution is uneven, and also an error in converting the total or average transmission of a heterogeneous object into an average extinction. It appears that, in direction and relative magnitude, these should run parallel with the distributional error which was just discussed (see Fano, 1947; Swift, 1950).

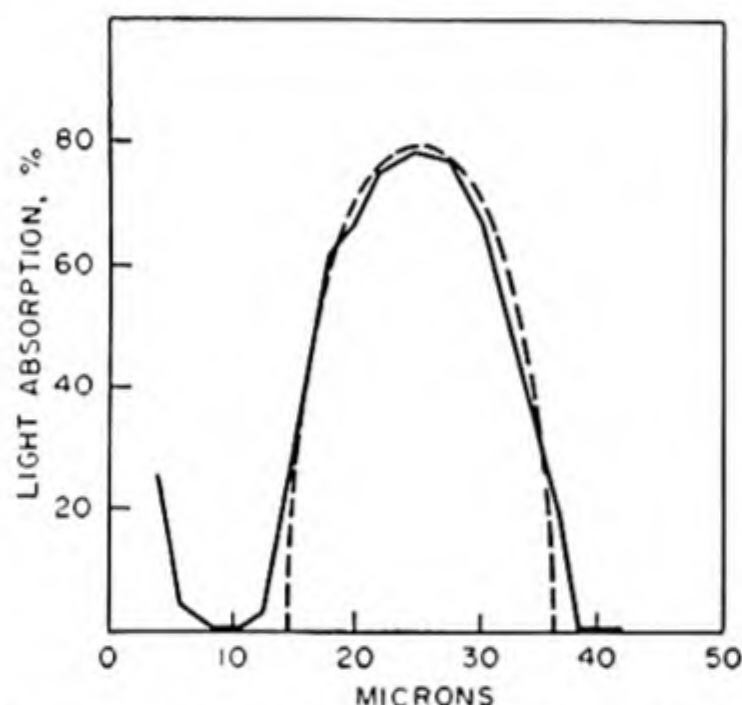


FIG. 6-11. Ultraviolet absorption measurements at a series of points across the diameter of a grasshopper spermatocyte (solid line), compared with the computed absorption curve of an absorbing sphere (broken line). (Redrawn from Caspersson, 1939.)

The unexceptionable method for determining amount of absorbing material in large areas with varying density is that of making densitometer cross-tracings of negatives (Caspersson, 1940a) or a series of scanings of a cell, in many azimuths, with the photometric apparatus (Thorell, 1947). Caspersson followed this procedure with living early meiotic prophase nuclei of the grasshopper; the cross-trace in one azimuth is shown in Fig. 6-11. He did not detect any distortion of what was to be expected from absorption of a sphere aside from some marginal diffraction, i.e., there was no measurable heterogeneity although, visibly,

there seemed some denser structures. Accordingly, he used the extinction through the center as a measure of concentration, and computed total amount in the nucleus from this datum.

Other possible sources of error in microspectrophotometry of cells are discussed in such references as Caspersson (1936, 1940a, b, 1950); Thorell (1947); Swift (1950, 1953); Naora (1951); Pollister (1952a); and Davies and Walker (1953).

#### 4-4. QUANTITATIVE APPLICATIONS, ABSOLUTE AND RELATIVE

Because of the potential errors in photometric analysis of cells it is evident that a straightforward computation of absolute amount of substance by referring light loss in a cell to any standard value obtained on

solutions in an absorption cuvette (as described in Sect. 2) is by no means a reliable procedure, although this has frequently been done.

The first such attempt at absolute quantitation was made by Caspersson (1939) who computed the amount of DNA in 24 nuclei of living grasshopper spermatocytes, assuming that distribution was homogeneous (p. 235 and Fig. 6-10) and that all light loss was due to specific DNA absorption. No correction was made for possible protein or RNA absorption, or for nonspecific light loss, but it now seems evident that these first two must have contributed to the total weakening of the light (Ris, 1947; Caspersson, 1950, Fig. 36).

It will be recalled that two kinds of ultraviolet absorption curves (curves I and II, Fig. 6-6) have been obtained for intracellular nucleoprotein. No absolute computations have been made from type I curves except the semi-quantitative nucleic acid-protein ratio, from extinctions near the nucleic acid and protein peaks (best described by Thorell, 1947). Caspersson, however, has made an elaborate effort to compute quantities of nucleic acid, tyrosine, and tryptophane from the type II curves, which were formerly supposed to characterize regions of nucleic acid accompanied by histone type or diamino acid-rich protein (Fig. 6-12). The method of computation has not been described completely enough so that readers may discover just how the values were reached from the measured curve. It is clear, however, that the curve analysis depends on the validity of the assumption that the protein moiety is of basic type in which the presence of a large proportion of dibasic amino acids, as in histones, brings about a shift of the absorption peak of tyrosine (as suggested very tentatively by Stenström and Reinhard, 1925). Therefore, this particular method of curve analysis is in effect invalidated by the demonstration that in histones the peak is not shifted toward longer wave lengths (p. 223).

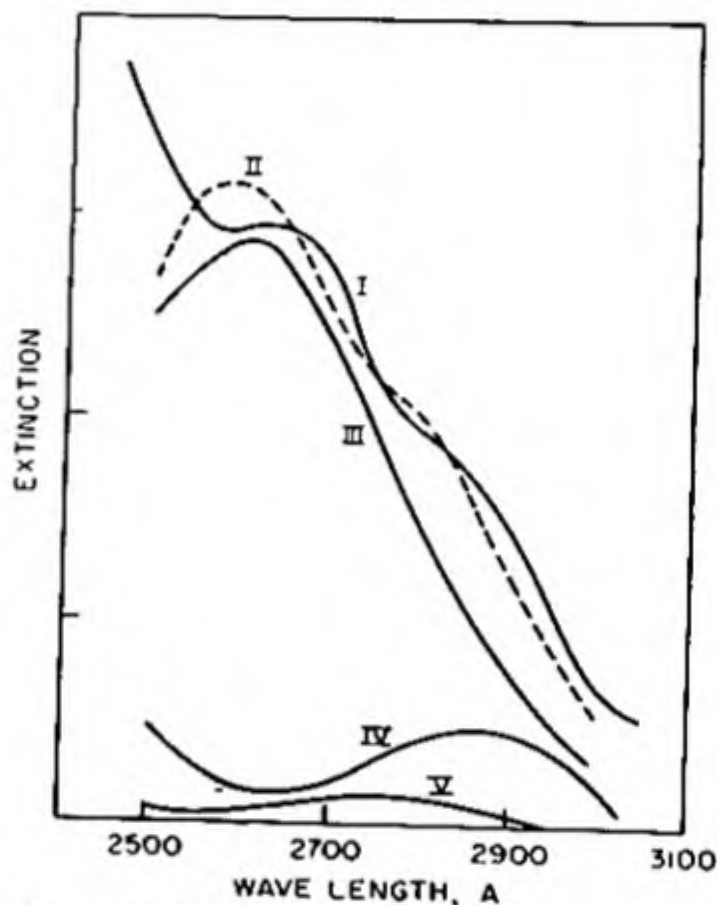


FIG. 6-12. An example of analysis of compound ultraviolet absorption curve of a cell structure containing nucleoprotein. Curve I, measured absorption; curve III, nucleic acid component; curve IV, tyrosine component; curve V, tryptophane component; curve II shows the sum of the components. Results: tyrosine, about  $0.1 \times 10^{-10}$  mg/ $\mu^2$ ; tryptophane,  $0.04 \times 10^{-10}$  mg/ $\mu^2$ ; nucleic acid,  $0.6 \times 10^{-10}$  mg/ $\mu^2$ . Light refraction and diffraction are, in this special case, negligible, as special experiments have shown. (Redrawn after Caspersson, 1940, 1950.)

Pollister and Ris (1947) reported computation of the amount of DNA



in isolated thymus nuclei, very much as Caspersson had done earlier for spermatocytes. They reported  $1.1 \times 10^{-9}$  mg per nucleus, and, for the first time in cytochemistry, compared a figure obtained by cytological photometry with one obtained by chemical analysis of a mass of known number of isolated nuclei. The two values agreed within 10 per cent, which appeared to be excellent validation of the microspectrophotometric procedure. It later appeared that this was merely a fortuitous combination of errors, since all other chemical studies indicated the amount per nucleus to be nearer  $6.0 \times 10^{-9}$  mg (see table in Davidson and Leslie, 1950b); hence the value computed by Pollister and Ris from ultraviolet absorption is probably considerably less than one-fifth the real amount.

This type of study was later carried out under much more favorable conditions by Leuchtenberger and coworkers (1951). The citrate-isolated nuclei were swollen in glycerin to minimize nonspecific light loss and to increase the homogeneity. The ultraviolet absorption was demonstrated by cross-scannings of the spherical nuclei, according to Caspersson's earlier procedure for spermatocytes (p. 237). The biochemists then determined the DNA, RNA, and protein content, and showed that the last two could have only slight effect upon the absorption. The amount of DNA per nucleus computed from these cytological data ( $5.4 \times 10^{-9}$  mg) is much closer to that found independently by the chemical analysis of the remainder of the same sample of isolated nuclei ( $6.1 \times 10^{-9}$  mg).

It must be pointed out that in such nuclei the DNA is determined under very special conditions of minimal RNA and protein, brought about by the citrate isolation. Such a condition is rarely, if ever, encountered in intact fixed tissue (see Pollister and Leuchtenberger, 1949a, Pollister, 1952b).

Di Stefano (1949) also computed DNA in frog cartilage nuclei from ultraviolet absorption data. By comparison with chemical analysis (Davidson and Leslie, 1950b), it appears that these values were deficient by about as much as those given for thymus nuclei by Pollister and Ris.

Di Stefano likewise compared the value from ultraviolet absorption with that computed from the Feulgen reaction, on the assumptions that at maximum reaction all purines had been removed and that the fuchsin regeneration from Schiff's reagent proceeded according to the scheme proposed by Wieland and Scheuing (1921). The DNA amounts per nucleus computed by these two cytological methods were in satisfactory agreement, but, as just indicated, these are far below the value obtained by chemical analysis. Protein-DNA ratios for tissue nuclei were computed from Feulgen and Millon reactions, before the invalidation of the Pollister and Ris and the Di Stefano attempts at absolute quantitation became known (Pollister and Leuchtenberger, 1949). The Feulgen data of the

last-named authors lead, by the Di Stefano type of computation, to DNA values about half those obtained by chemical analysis of isolated nuclei (see Pollister, Swift, and Alfert, 1951).

As this brief summary shows, in practice the computation of absolute amounts from microscopic absorption measurements has been far from uniformly successful, a result not unexpected in view of the many potential errors. It is apparent, however, that for many problems in cell chemistry this dubious extrapolation is not necessary; an adequate answer is

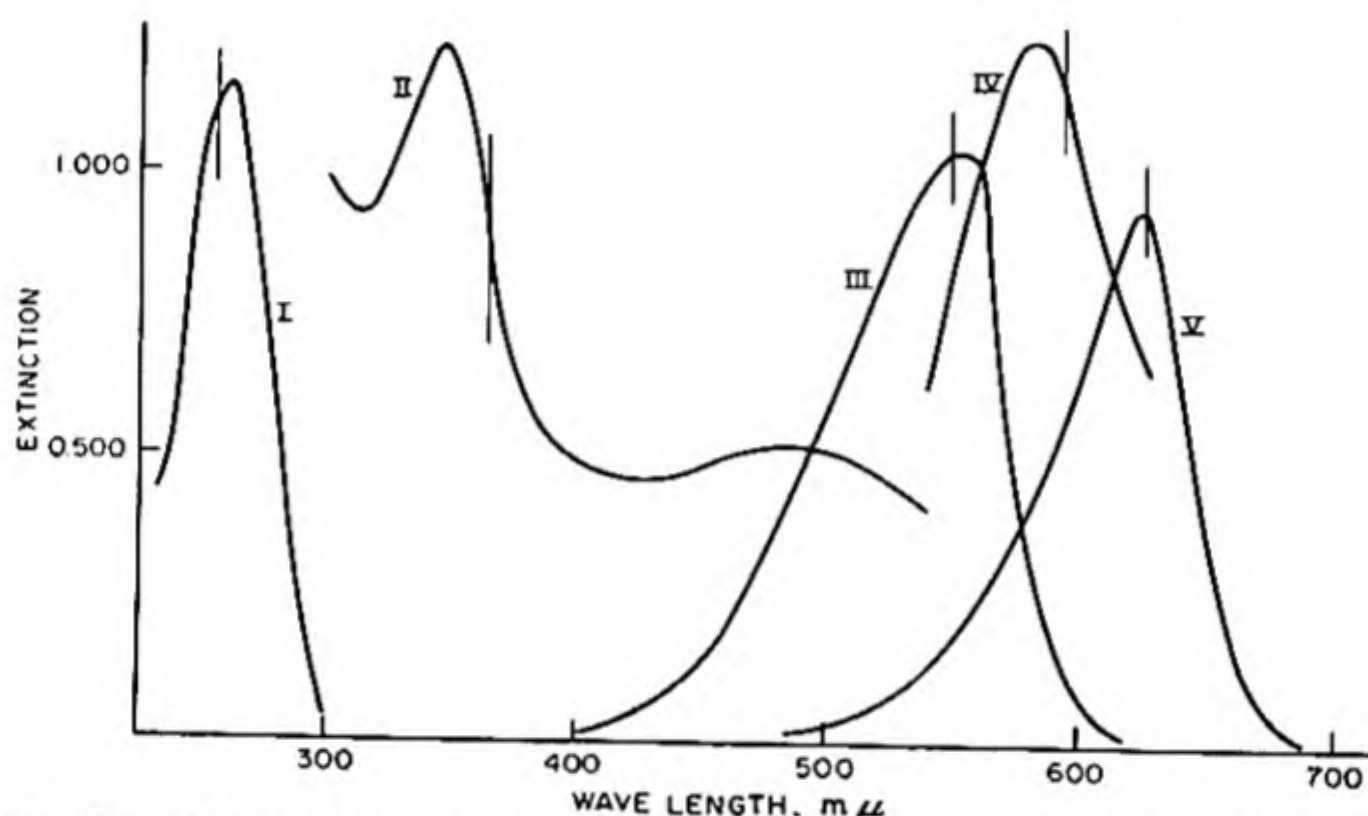


FIG. 6-13. Absorption curves, measured in a Beckman spectrophotometer, of various substances which are analyzed photometrically in cytological preparations. Unless otherwise indicated, the length of the absorption path was 1 cm. The light vertical line cutting each curve indicates the spectral region which is measured in the microscopical preparations. Curve I, natural absorption of deoxyribonucleic acid, concentration about 0.05 mg/cc (*redrawn from Pollister and Mirsky, 1946*); curve II, tyrosine-mercurial formed by the Millon reaction, tyrosine concentration 0.045 mg/cc; curve III, fuchsin regenerated from standard Schiff's reagent by addition of formalin; curve IV, azure A in distilled water, concentration 0.5 mg/cc, thin cuvette (*data of M. Flax*); curve V, methyl green (Natl. Anil., cert. No. NG35) in carbol-glycerin-alcohol mixture, concentration 0.01 mg/mm<sup>3</sup> (*data of C. Leuchtenberger*).

obtainable if the question of relative amount or of relative change can be answered. Numerous quantitative studies with this aim have been published, some of which are reviewed in Pollister (1952a). The specific methods are those which have already been discussed in Sect. 3. The *in vitro* absorption curves and the wave lengths measured in microscopic material are shown in Fig. 6-13. Since these methods are colorimetric (that is, measurement at a single wave length) and the apparatus is simple (Fig. 6-7), large numbers of absorption measurements on individual nuclei, nucleoli, or other cell structures are readily obtained. On any one type of structure the values are found to vary considerably, the highest extinctions being often as much as twice the lowest. When



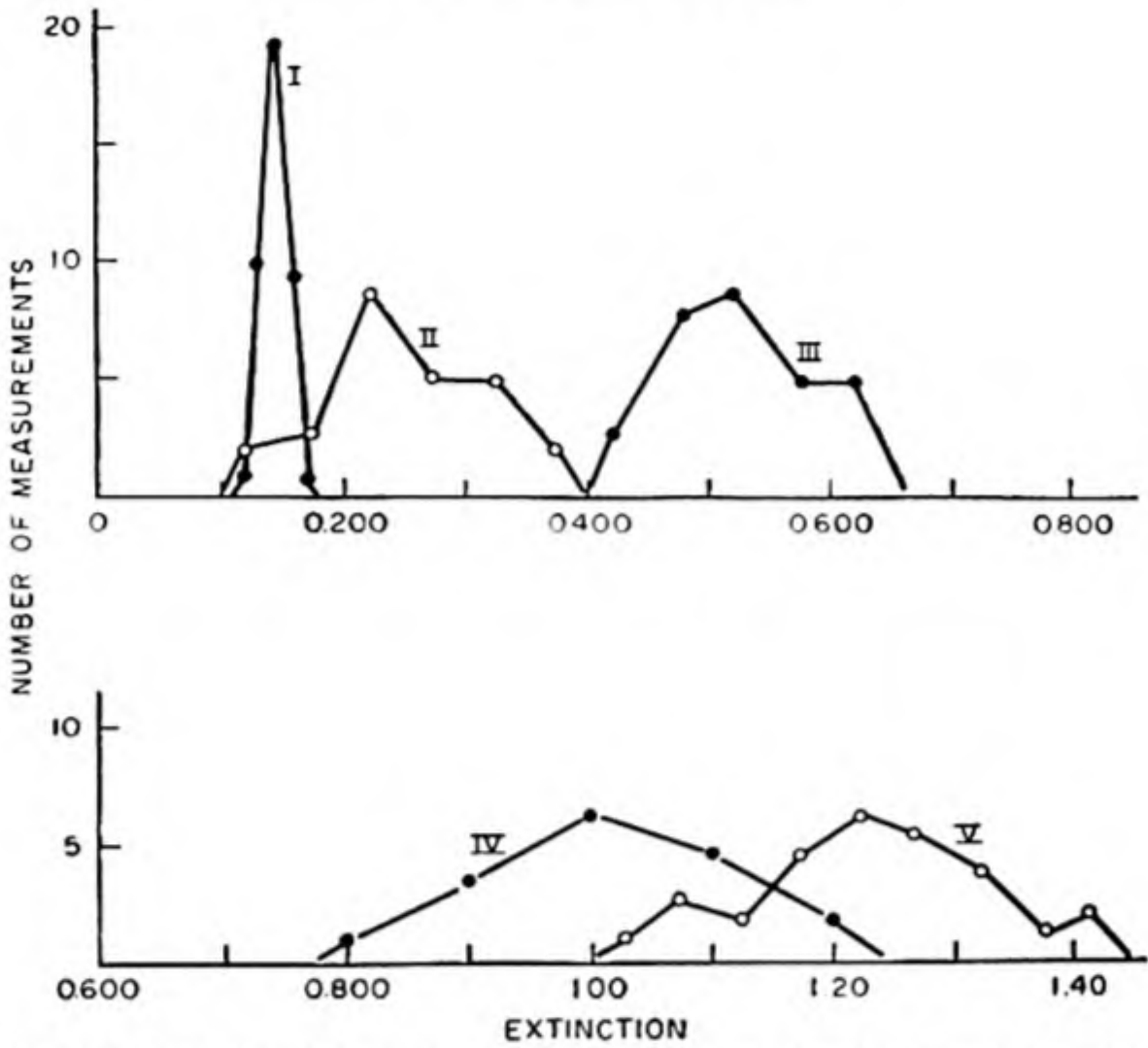


FIG. 6-14. Typical distribution curves of microspectrophotometric data. Curve I, sections of nucleoli, corn pollen mother cells, Millon reaction; curve II, nuclei of mouse sarcoma, methyl green; curve III, nucleoli, corn pollen mother cells, ultraviolet absorption; curve IV, mouse spermatocyte nuclei, Feulgen reaction; curve V, preleptotene nuclei from mouse testis, Feulgen reaction. (After Pollister and Swift, 1950.)

TABLE 6-5. LIVER NUCLEI, ULTRAVIOLET ABSORPTION

Slide no.	Number measured	Mean $E_{254}$
A-6	20	$0.489 \pm 0.015$
A-6	10	$0.474 \pm 0.024$
A-4	20	$0.466 \pm 0.023$
A-9	22	$0.507 \pm 0.016$
A-8	25	$0.475 \pm 0.012$
A-5	23	$0.499 \pm 0.027$
		Mean = 0.485

The reproducibility of ultraviolet absorption of 5.0- $\mu$  sections of nuclei of the liver of the salamander, *Amblystoma*, is shown by data from different slides of the same organ, measured at different times. Fixation in acetic acid-alcohol, embedded in paraffin, mounted in glycerin, measured as absorption of 254 m $\mu$  radiation isolated from a Hanovia SC2537 mercury-vapor lamp by a Zeiss two-prism quartz monochromatic illuminator, with glycerin immersion monochromatic objective, N.A. 0.85, condenser aperture about N.A. 0.40.

enough data are accumulated for statistical analysis it is found that these values group themselves into normal unimodal distribution curves (Fig. 6-14). The cause of this variability is not fully known in any instance. The instrumental and cytological variability can account for but a small part; in many structures (e.g., interphase nuclei) a major portion appears to be due to the distributional error. Until these errors of technique and unavoidable errors due to cytological structure can be precisely estimated (Sect. 4-3) the magnitude of the biological variation in any one population of similar cells cannot be estimated; and therefore many of the possible more subtle correlations of nucleoprotein composition with physiological, developmental, pathological, or experimental phenomena cannot be investigated by microspectrophotometry. In the meantime, it has been found possible to make many fruitful studies by utilizing the fact that with proper cytological and photometric techniques the mean values of any population are reproducible to about 10 per cent. This holds when comparing different slides of one material (Table 6-5), when examining the same animal at different times (Table 6-6), or when studying different animals of the same species (Table 6-6). This variability of the means presumably is a measure of the over-all error of the technique of preparing tissues for microscopic examination and of the photometric procedure. In this type of relative quantitative analysis, a major change of composition which may accompany cyclical, experimental, or pathological events is detected as a change of mean value, outside the normal range of variability. This reproducibility of the means has been shown repeatedly for each of the techniques indicated in Fig. 6-13; indeed, it is customary to reexamine it for each new material studied.<sup>4</sup>

An important aspect of these relative quantitative studies is that in many cases it has been possible to compare them with results by independent methods, such as biochemical analysis of samples of known numbers of isolated cell components. For example, the correlation of amount of DNA with the chromosome complement of nuclei, first indicated by biochemical analysis, has been confirmed and greatly extended by cytological studies made with the Feulgen reaction, whereas the protein composition of the nucleolus, suggested by results with the Millon reaction, has been confirmed by both X-ray absorption data and analysis of masses of isolated nucleoli. Many of these essential validations of the cytochemical analyses are summarized in Pollister (1952a).

<sup>4</sup> The approach of the Caspersson school to microspectrophotometry has been almost entirely qualitative and semiquantitative. This is emphasized particularly by the fact that in the total of nearly a hundred publications (see list in Caspersson, 1950) there is not one demonstration of the statistical reproducibility of the ultraviolet absorption technique, nor is the urgency and priority of such a demonstration ever recognized.



TABLE 6-6. REPRODUCIBILITY OF FEULGEN REACTION AND METHYL GREEN BASOPHILIA  
(Reisner and Korson, 1951.)

Case no.	Diagnosis	Feulgen, $E_{550}$			Methyl green, $E_{625}$		
		No. of nuclei	Before treatment	No. of nuclei	After treatment	No. of nuclei	Before treatment
1	Normal	30	0.231 $\pm$ 0.003	...	.....	20	0.138 $\pm$ 0.003
2	Nutritional macrocytic anemia	20	0.250 $\pm$ 0.009	30	0.247 $\pm$ 0.007		
3	Megaloblastic anemia of infancy	30	0.244 $\pm$ 0.007	5	0.244 $\pm$ 0.008		
4	Pernicious anemia	10	0.232 $\pm$ 0.007	20	0.242 $\pm$ 0.005		
5	Pernicious anemia	35	0.249 $\pm$ 0.005	27	0.238 $\pm$ 0.005		
6	Pernicious anemia	30	0.240 $\pm$ 0.004	35	0.240 $\pm$ 0.005	20	0.136 $\pm$ 0.003
7	Juvenile pernicious anemia	30	0.231 $\pm$ 0.005	40	0.237 $\pm$ 0.006	11	0.131 $\pm$ 0.005
8	Juvenile pernicious anemia	50	0.248 $\pm$ 0.005	20	0.242 $\pm$ 0.006	5	0.148 $\pm$ 0.003
9	Pernicious anemia	35	0.231 $\pm$ 0.006	20	0.235 $\pm$ 0.007	12	0.146 $\pm$ 0.003
10	Pernicious anemia	40	0.256 $\pm$ 0.006	30	0.248 $\pm$ 0.005	15	0.140 $\pm$ 0.004
	Total	310		227		83	
	Mean		0.241		0.241		0.140
						90	0.143

Mean extinction values and standard error of the mean for Feulgen and methyl green-stained erythroblast nuclei, all 9.5  $\mu$  in diameter, on smears from human marrow aspirates, before and after administration of vitamin B<sub>12</sub>.

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## CHAPTER 7

# The Effect of Ultraviolet Radiation on the Genes and Chromosomes of Higher Organisms

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*Introduction. Experimental procedures. Genetic effects of ultraviolet radiation. Direct effects of ultraviolet radiation on chromosomes. Spectral relations. References.*

### INTRODUCTION

This review will be concerned with the effects of ultraviolet radiation on the genes and chromosomes of organisms above the microbiological level. The group of organisms thus included is relatively small in numbers, and the literature is scanty in comparison to that devoted to radiation studies on the fungi, bacteria, and viruses. But cytogenetic studies can be made only on organisms with suitable chromosomes. The ultraviolet results obtained with *Drosophila melanogaster* and *Zea mays* may be evaluated against an extensive background of X-ray data bearing on problems of cytogenetic interest. Supplementing the data from these organisms are those from *Antirrhinum* and *Sphaerocarpus*, which relate to the genetic effectiveness of various wave lengths within the ultraviolet spectrum, and those from *Tradescantia* and *Gasteria*, which deal only with induced chromosomal aberrations.

Each of these species possesses certain disadvantages, none being wholly satisfactory as a test organism because of difficulties of radiation penetration, accurate dosage measurements at the site of genetic alteration, or critical analysis of induced effects. Despite these shortcomings, however, the accumulated evidence from ultraviolet studies has been

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sufficient to advance materially the understanding of the nature of induced hereditary changes.

The genetic action of ultraviolet radiation bears on three major problems. The first of these is the analysis of the complex of genetic variations induced by the ionizing radiations. It is invariably found, where adequate cytogenetic tests can be made, that both mutations and chromosomal aberrations are induced by X radiation. A clear-cut separation of the two phenomena has not yet been accomplished, and the question of their similar or dissimilar nature and origin remains unanswered. If the induction of mutations and chromosomal rearrangements by the ionizing radiations results from some common, fundamental effect, the two types of genetic alteration should have a common spectral limit. The analysis of induced hereditary variations would then involve determining the alternate pathways of reaction which culminate in a variety of genetical and cytological expressions. If, on the contrary, the diverse effects of X radiation are of independent origin, it is possible that their spectral relations may be sufficiently different to permit the separation and perhaps the selective induction of one type of variation to the exclusion of others. The individual phenomena should then prove to be more amenable to analysis.

The second problem is concerned with the determination of genetic effectiveness of specific wave lengths of the ultraviolet spectrum, as a clue to the chemical nature of the substance within which the energy absorption leading to genetic change takes place. With ionizing radiations such a study is not possible since their absorption is independent of molecular organization.

The third problem is concerned with the nature of mutations in general, whether spontaneous or induced. Mutations as experimentally identified are a residual class, identified by negative criteria. The analysis of specific X-ray-induced mutations has shown that character changes inherited as if due to gene mutations may be in some instances the result of chromosomal rather than genic alteration. The category "mutations," as experimentally defined, must therefore be a complex one, including various extragenic as well as intragenic alterations. Since the mode of action of ultraviolet radiations is so different from that of ionizing radiations, the comparative study of mutations induced by these agents is promising. The relative infrequency of chromosomal derangements induced by ultraviolet suggests that certain extragenic alterations simulating gene mutation may be infrequent or absent among the mutations from ultraviolet treatment. The possibility of qualitative differences in the chromosomal alterations must also be considered.

The spectral analysis of the complex of genetic effects induced by X rays, as outlined above, implies the assumption that the mutations and chromosomal alterations induced by X rays are qualitatively analogous

to those induced by ultraviolet radiation. If they are not, it is possible that the spectral limit of both classes of X-ray alterations is far below the shortest ultraviolet wave length that can be used in biological experiments, and that the mutations and chromosomal alterations characteristic of the ultraviolet are of a type not occurring in appreciable frequency in the X-ray progenies.

### EXPERIMENTAL PROCEDURES

Genetic and cytological studies of the effects of ultraviolet radiation on higher organisms are limited by the techniques which permit the investigator to irradiate the germ cells effectively without killing the organism or the cells being treated. Ultraviolet produces considerable physiological damage, setting limits to the dosage; it is also low in penetrating power, a disadvantage when deep-seated sex organs are being treated. Both of these phenomena result from the high absorption of ultraviolet by extranuclear constituents of the cells as well as by the nucleoproteins of the chromosomes. A number of techniques are available, however, and it is fortunate that they permit the irradiation of the germ cells of *Drosophila* and maize, thus making possible genetic and cytological comparison with other mutagenic agents. Ultraviolet radiation and X rays have also been used extensively with *Neurospora*, in which significant cytogenetic comparisons could be made, but no comparative studies of the induced alterations have been reported.

Perfection of the technique of artificial insemination of *Drosophila* females with treated spermatozoa would circumvent the difficulties of reaching the sex cells of adult males with ultraviolet radiation, but apparently all attempts to duplicate Gottschewski's (1937) artificial insemination results have so far been unsuccessful. Two other methods, however, have been developed: (1) irradiation of the pole cells of the early embryo in the egg, and (2) irradiation of mature spermatozoa through the ventral side of compressed abdomens of adult males.

The polar cap technique was developed by Geigy (1931) after the early work of Guyenot (1914) and Altenburg (1928) had demonstrated that ultraviolet treatment of adult males gave only inconclusive results. The pole cells are destined to enter the germ tract. At 75 min after fertilization of the egg (at 24°C) the pole cells appear close to the surface at the amicropolar end, where they remain for approximately 1 hr (Altenburg, 1934). In this position their nuclei can be readily reached by ultraviolet radiation. The early experiments of Altenburg (1933, 1934) were made with the angle of incidence of the ultraviolet at right angles to the vertical axis of the egg, the lower portion of which could be shielded to reduce injury to the developing embryo. More recently, it has been found that a shift in the angle of incidence from a plane of 90° to one more nearly



parallel to the axis of the egg greatly increases the genetic effectiveness of a given ultraviolet dose while lessening the physiological damage to the embryo (Altenburg *et al.*, 1950; Meyer *et al.*, 1950). Dechoriation of the eggs by immersion in a 5 per cent solution of sodium hypochlorite also facilitates penetration of the radiation (Clark, 1948).

At the time of irradiation, the pole cells number about 20. Their later incorporation into the germ tract is accompanied by an increase in the number of cells as the sex organs and the reproductive cells are formed. Therefore, if a mutation is induced in one gene at the polar cap stage, it theoretically should appear replicated in about 5 per cent or more of the sex cells from that particular individual. Should the mutation occur after the process of multiplication has begun, a smaller proportion of the germ cells would receive it.

Two assumptions are involved if the 5 per cent level of mutation replication among the  $F_1$  offspring provides the distinction between induced and spontaneous changes. In the first place, the pole cells at the time of exposure to radiation must be 20 or less in number, and second, an equal rate of multiplication of mutated and normal pole cells must be assumed to take place up to the time of formation of the reproductive cells. Unequal exposure of the pole cells through unavoidable shielding as well as through differences in the degree of penetration of radiation would suggest that variations in cellular injury are to be expected. These variations would be expressed in unequal rates of cell multiplication, with the most heavily injured cells having the slowest rate of division. The latter cells would also be most likely to possess mutated genes. As a means for demonstrating that ultraviolet is effective in inducing mutations, the technique is entirely satisfactory, but it is unsuitable for accurate determinations of the frequency of mutation as a function of dosage.

Recessive lethals induced in the X chromosome by the pole cap method of exposure may be determined by testing, through the ClB technique, the  $F_1$  daughters of males arising from irradiated eggs. Replicated lethals from any single male must be further tested for identity because of the possibility of two or more coincident lethals. Visible mutations in the X chromosome may be detected in  $F_1$  males by breeding the treated  $P_1$  males to  $\widehat{XX}$  females.

Reuss (1935) developed an effective method for the exposure of mature spermatozoa to ultraviolet radiation. The abdomens of adult male *Drosophila* are compressed gently between quartz plates, the radiation being applied ventrally. Since the testes are superficially located, the amount of overlying tissue is at a minimum and consists of the chitinous exoskeleton, the wall of the testis, and the intervening connective tissues. Clear areas of chitin, which has chemical and absorptive properties characteristic of polysaccharides, are in general highly penetrable by wave lengths from 250–400  $m\mu$ , with the degree of absorption increasing rapidly

at shorter wave lengths (Durand *et al.*, 1941). The method, while useful in ascertaining the kinds of gene and chromosome changes which may be induced by ultraviolet in the mature spermatozoa, is not suitable for the accurate quantitative determination of dosage-mutation frequency relations and wave-length dependence yields. The chitin is of uneven transparency and the intervening tissues vary in thickness and position, with the result that considerable differences in the amount of radiation reaching the spermatozoa must be expected from one individual to another. The variations in penetration of radiation can be lessened somewhat by the use of light-colored mutant stocks, but there remains the difficulty of accurately determining the amount of energy absorbed by the sex cells at the site of effective action. Also, the practical limits of dosage are determined largely by the tolerance of the adults to physiological damage leading to sterility. Demerec *et al.* (1942) have reported induced sex-linked recessive lethals in frequencies as high as 50 per cent, but such high proportions of mutations are a rare exception. The usual frequencies of recessive lethals obtained from optimal abdominal exposures range from 0-5 per cent, with a wide variation among similarly treated flies.

The higher plants offer somewhat better technical possibilities for studies of the genetic effects of ultraviolet radiation. The male cells are of minute size, readily accessible, and free from extraneous tissues. Three types of cells have been used: (1) swimming spermatozoids, (2) pollen grains, and (3) generative cells in developing pollen tubes.

Swimming spermatozoids of the liverworts, for example those of *Sphaerocarpus donnellii*, are particularly favorable objects for ultraviolet studies, since they consist essentially of a naked nucleus approximately  $0.5 \mu$  in diameter (Knapp, 1938). Absorption of the radiant energy by extranuclear materials is at a minimum, and treatment of the cells can be carried out in water. Their use in the determination of dosage relations has not been exploited to the extent warranted by the excellence of the material.

Exposure of the male cells of angiosperms can be readily made by treating a monolayer of loose, dry pollen. Pollens of maize, *Antirrhinum*, and *Gasteria* have been successfully employed in ultraviolet studies, the irradiated grains being used to fertilize untreated plants. The maize pollen grain has both sperm cells fully formed at the time of anthesis. One sperm fertilizes the egg to produce the zygote while the other unites with the fusion nucleus to give rise to the endosperm. Maize also possesses the added advantage of a goodly number of clear-cut endosperm marker genes whose presence or absence (or mutated state) can be directly determined by examination of the kernels produced on ears pollinated by the treated pollen. These markers facilitate the collection of massed data and provide a convenient measure of genetic effectiveness when comparative studies are being made of wave-length and dosage



relations. In both maize and *Antirrhinum*, mutation data may be obtained through the detection of segregating characters in  $F_2$  populations. *Gasteria* has been used only for the study of induced chromosomal changes appearing in the cells of  $F_1$  embryos.

Compared with the spermatozoids of liverworts, the pollen grains of angiosperms are relatively large, those of maize, for example, being almost  $100\ \mu$  in diameter. When comparisons are being made of the relative effects of different wave lengths or of different doses of the same wave length, it becomes necessary to take into account the factor of internal filtration, since the energy incident at the surface of the pollen grain is very greatly reduced by absorption in the extranuclear material. Internal filtration varies greatly with the wave length of radiation employed (Uber, 1939; Stadler and Uber, 1942), and failure to correct for these differences of penetration may lead to gross error in wave-length comparisons. The filtration factor can be roughly calculated, as Stadler and Uber have shown, but the difficulties involved stress the need for better genetic materials in this area of investigation.

Cytological studies of ultraviolet-irradiated chromosomes have been carried out in the  $F_1$  progeny of maize, *Gasteria*, and *Drosophila*. The chromosomes of surviving  $F_1$  individuals represent a selected group from which all inviable aberrations have been screened. Through genetic techniques, the types of chromosomal rearrangements induced by ultraviolet may be inferred without cytological examination, but there remains the possibility that certain aberrations may be eliminated after the passage of several cell generations. The pollen-tube technique overcomes this difficulty in that it permits a direct examination of irradiated chromosomes before the elimination of inviable changes can take place. The technique involves the culturing of pollen tubes on an agar-coated slide, with sucrose or lactose added to the agar as a carbon source. The generative cell, after passing from the pollen grain into the tube, is covered only by a thin cytoplasmic layer and a thin tube wall. Since the pollen tube is narrow (approximately  $5\ \mu$  in *Tradescantia*), the amount of radiation absorbed before it reaches the nucleus is not great. The chromosomes, undergoing mitotic division in the tube, may therefore be readily exposed to ultraviolet, and an analysis of structural changes may be made at metaphase by blocking the division with colchicine (Swanson, 1940, 1942). The method, as first employed with *Tradescantia*, has been materially improved by Bishop (1949). Certain limitations in the technique must be recognized, however, if the derived data are to be logically compared with those obtained from other organisms. In the first place, cytological analysis is made on the heavily condensed metaphase chromosomes; small aberrations such as interstitial deficiencies, if present, are quite likely to pass unnoticed. Second, the chromosomes cannot be maintained and studied beyond the metaphase stage. Any aberrations,

therefore, which are realized only at a later stage in cell division would not be detected. The absence of ultraviolet-induced translocations in the pollen tube chromosomes of *Tradescantia*, as contrasted to their occurrence in the  $F_1$  populations of maize and *Gasteria*, may well be due to the formation of these aberrations at later stages of division or during the process of fertilization.

### GENETIC EFFECTS OF ULTRAVIOLET RADIATION

Guyenot's (1914) early attempt to induce mutations with ultraviolet radiation was unsuccessful. Adequate techniques for the quantitative screening of mutations were not available at the time, and genetic knowledge was too scanty to provide a background against which such studies could be properly evaluated. After Muller's discovery (1927) of the significance of X rays as a mutagenic agent, the question of the spectral limits of genetic effectiveness arose. Genetic studies with ultraviolet radiation by Altenburg (1928) on *Drosophila* and Stubbe (1930) on *Antirrhinum* gave no positive indication that this radiation could induce mutation. As the techniques of irradiation were improved, however, it became apparent that under favorable conditions of exposure mutations could be induced in both plants and animals. Early indications of effectiveness were reported by Altenburg (1930, 1931), Geigy (1931), and Promptov (1932) in *Drosophila*. Largely as a result of the development of the polar cap technique of exposure by Geigy, unequivocal confirmation of the mutagenic action of ultraviolet in *Drosophila* was provided by Altenburg (1933, 1934). Noethling and Stubbe (1934) also demonstrated in *Antirrhinum* that exposure of the pollen grains to ultraviolet could significantly increase the mutation frequency.

*Experiments with Drosophila.* A summary of Altenburg's *Drosophila* data is given in Table 7-1. From these figures it is clear that a significant increase in the frequency of recessive lethals can be obtained by exposure to ultraviolet. As might be expected on the basis of penetration, eggs in the polar cap stage are readily affected. The replicated lethals appearing in 5 per cent or more of the  $F_1$  females from a single male represent mutations occurring in pole cells at the time of treatment, and their frequency is clearly increased by irradiation. The distinct increase in "isolated" lethals (i.e., lethals that appear singly) is assumed to be due, at least in part, to the inclusion of some eggs beyond the polar cap stage at the time of treatment.

The results of Geigy (1931) and Promptov (1932), on the induction of recessive lethals in eggs of *Drosophila*, are in essential agreement with those reported by Altenburg (1934). Both Altenburg and Promptov noted an increased incidence of visible mutations, in frequencies considerably lower than for sex-linked lethals.



Each of the 79 lethals recorded in Table 7-1 was tested for position on the X chromosome and found to be unaccompanied by disturbances of crossover frequency with neighboring loci. The following conclusion was drawn: "Ultraviolet light therefore produces no inversions or other chromosomal changes that are detectable from changed crossover values. In this respect, the effect of ultraviolet light differs markedly from that of X rays" (Altenburg, 1934).

TABLE 7-1. SUMMARY OF ALTENBURG'S (1934) *Drosophila* DATA IN WHICH ULTRAVIOLET RADIATION WAS SHOWN TO BE MUTAGENIC IN NATURE

Stage treated	Number treated	Types of recessive lethals in F <sub>1</sub> offspring			Total lethals
		Induced in 5% or more	Spontaneous in 5% or less	Isolated	
Adults:					
Control.....	8694	..	..	..	2
Treated.....	9239	..	..	..	24
Larvae:					
Control.....	3094	..	..	..	1
Treated.....	3098	..	..	..	7
Eggs:					
Control.....	222 <sup>a</sup>	1	2	13	16
Treated.....	239 <sup>a</sup>	13	3	32	48

<sup>a</sup> From the 222 control eggs, 13,063 F<sub>1</sub> females were tested; from the 239 treated eggs, 14,059 F<sub>1</sub> females were tested.

The development of the abdominal exposure technique by Reuss (1935) demonstrated the feasibility of inducing mutations in the mature spermatozoa of *Drosophila*. Mackenzie and Muller, using this method, have confirmed and extended the earlier findings of Altenburg, particularly as concerns the comparison of the effects of ultraviolet and X rays (Muller and Mackenzie, 1939; Mackenzie and Muller, 1940; Mackenzie, 1941). The filtered radiation employed consisted of wave lengths above 280 mμ, a quality of radiation less damaging physiologically than the shorter wave lengths. A dose of 2 × 10<sup>5</sup> ergs/mm<sup>2</sup> was found to be optimum for the study, since an appreciable frequency of sex-linked lethals (about 3 per cent) was induced without an accompanying high degree of sterility or mortality. Higher doses raised the frequency of lethals to 9 per cent, but the sterility was disproportionately increased, making extensive observations difficult and quantitative comparisons unreliable. The principal results of these studies were the following:

1. No translocations affecting the Y, II, and III chromosomes were found in a population which had a frequency of sex-linked lethals of 4.3 per cent. Such a frequency would be induced by an X-ray treatment of about 1300 r. The authors estimate that, in the number of cultures

tested, 40 or more translocations of type II-III alone would have resulted from this X-ray dose. These data were in agreement with an earlier trial in which no translocations were found from treatments which induced sex-linked lethals in such numbers as to indicate an expectancy of at least 25 detectable translocations, on the basis of the relation found with X rays. The discriminatory action of the ultraviolet was thus shown by the absence of detectable translocations in cultures in which at least 65 were to be expected if the relation of mutation and gross chromosomal rearrangement were the same with ultraviolet as with X rays.

2. Similar evidence relating to the occurrence of minute rearrangements was obtained by the use of a special technique, with which minute rearrangements are induced by X rays in relatively large numbers and are recognizable by mutants at specific loci. In ultraviolet-treated cultures yielding sex-linked lethals at a rate corresponding to an X-ray dose of 1000 r, no mutants at these loci were found. In an X-ray experiment by Muller and Makki (Mackenzie and Muller, 1940), these had occurred at significant frequencies following dosage of 1000 r. Muller and Mackenzie concluded provisionally that the ultraviolet does not produce minute rearrangements, or at least that it is far less efficacious in this respect than X rays.

3. Wave lengths above 320  $m\mu$  were found to be ineffective in inducing mutations.

4. The frequency of mutations was higher when mating of the irradiated males followed immediately after treatment. No mutations were transmitted 5 days after irradiation although the supply of mature sperm would not have been exhausted for some days thereafter. Those irradiated flies which bred but died early had higher frequencies of mutations than those which continued to breed over longer periods of time. A correlation was established therefore between the frequency of induced mutations and the amount of physiological damage as determined by the duration of the fecund period. Both phenomena are undoubtedly affected by the degree of penetration of ultraviolet, a factor which varies widely among similarly exposed individuals.

5. Dose fractionation was without effect on the frequency of mutations or the degree of sterility.

The occurrence of minute deletions among the mutants induced by ultraviolet was cytologically demonstrated by Slizynski (1942). Among 21 of the sex-linked lethals produced in the experiments of Mackenzie and Muller, 5 were found to be cytologically detectable deficiencies, 1 involving the loss of 1 band, 3 the loss of 2 bands, and 1 the loss of 14 bands. All were interstitial deficiencies.

Results of studies by McQuate (1950) support the hypothesis of Mackenzie and Muller that terminal deficiencies, if produced, are not recovered in *Drosophila* populations derived from mature spermatozoa



which have been exposed to ultraviolet. Using a stock containing a special Y chromosome ( $y^3 \cdot Y^1$ ) marked with the normal allele of *achaete*, he mated irradiated males to *achaete* females. Terminal loss of the normal allele of *achaete*, with retention of the remainder of the special Y chromosome, would yield fertile *achaete* males in the  $F_1$  population. From a total of 19,309  $F_1$  males, 23 sterile *achaete* exceptions were found. Such males, however, result from a loss of the paternal X chromosome, or of all or part of both arms of the  $y^3 \cdot Y^1$  chromosome, losses which could arise through lagging of the chromosomes in division, or by breakage followed by fusion of broken ends to give acentric and dicentric portions having a low survival probability. Breakage of the  $y^3 \cdot Y^1$  chromosome, with loss of the  $y^3$  region (which also includes the normal allele of *achaete*) and followed by healing, did not occur. The fertile *achaete* exceptions, two in number, were no more frequent than in the control population.

*Experiments with Plants.* In many species of plants the pollen grain may be effectively treated with ultraviolet radiation. It is therefore feasible to make somewhat simpler tests of the genetic effects of the treatment than can be made in the experiments with *Drosophila*. The indirect analysis required by the polar cap technique is avoided, and the difficulties from internal filtration, while serious, are not nearly so great as in the irradiation of the sperm within the body of the adult fly. In the cultures grown from seeds produced by the use of the irradiated pollen (which we may, for convenience, refer to as the  $F_1$  cultures), each plant provides the material for testing the effects of the treatment on one irradiated gamete. Dominant effects of chromosome or gene changes induced by the treatment may be observed in the  $F_1$  plants, and each  $F_2$  culture produced by self-fertilization of one of these plants shows segregation for any haplo-viable recessive alteration induced in the gamete tested.

The results reported by Noethling and Stubbe (1934) clearly demonstrated the effectiveness of ultraviolet in inducing mutations. These were detected in segregating  $F_2$  populations of *Antirrhinum*.

A similar increase in the frequency of point mutations was found in maize by Stadler and Sprague (1936a), together with evidence of certain chromosomal effects of the treatment and further indications of differences in the genetic action of ultraviolet radiation and X rays. The mutations identified were only those affecting seed and seedling characters. Progenies representing various doses of unfiltered ultraviolet radiation yielded 31 mutations from 830 gametes tested, and control progenies yielded 6 mutations from 557 gametes.

This increase in mutation rate, while clearly significant, is not large. But among the 31 mutants detected (of which 9 would have been expected without irradiation), there were two cases in which 2 unlinked mutants occurred in a single  $F_2$  progeny, and one case with 3 unlinked mutants in a single progeny. These represent cases of two or three presumably unre-

lated mutants in a single treated gamete. In addition, three of the other mutants occurred in the progeny of plants segregating for defective pollen. The independent inheritance of the mutants, in these and several similar cases, has been shown by Sprague (1942). The degree of coincidence is far beyond that expected by chance, if all the tested gametes received an equal dose. But the treated gametes must receive quite unequal doses, for the sperm nuclei are eccentrically located in the spheroidal pollen grains, and the loss by internal filtration must vary widely with the casual orientation of the individual grains. If this is the explanation of the coincidences observed, the frequency of mutation in the most effectively exposed pollen grains must be very high. In later experiments with more effective ultraviolet treatments, mutation rates of about 20 per cent have been reported (Stadler, 1941b). These are subject to the same limitation by casual orientation of the pollen grains treated, and the results suggest that the effect of ultraviolet radiation on mutation frequency in maize, in individuals that are effectively treated, may be well beyond that produced by X rays.

A technical advantage of the maize material is the availability of many endosperm characters of known inheritance. In matings with appropriate marker genes, the loss of the effect of dominant alleles present in the male parent may be detected at once by phenotypic changes in the endosperms of the seeds produced. Linked endosperm characters, determined by genes located on one arm of one of the chromosomes, permit the detection of deficiencies in this region.

Ultraviolet treatment applied to the pollen greatly increased the frequency of endosperm deficiencies, as detected by loss of the linked factors *C* and *Wx*. The frequency of loss of other dominant genes for endosperm characters (*A*, *Pr*, *Su*) was similarly increased. There are no linked genes for endosperm characters suitable for determining whether *A*, *Pr*, and *Su* losses represent deficiencies. The fact that ultraviolet-induced loss of *C* and *Wx* is usually coincidental indicates that the endosperm effect is due usually, if not always, to deficiency rather than to gene mutation. Since no genetic analysis can be made, the identification of an individual case as a recessive mutation rather than a deficiency could not be positively established. The cases are referred to as "endosperm deficiencies," with the reservation that there may be included among them an unknown proportion of losses of dominant characters as a result of gene mutation.

The endosperm deficiencies resulting from ultraviolet treatment of pollen included a large proportion of fractionals, in contrast to those resulting from X-ray treatment of pollen, which are largely deficiencies affecting the entire endosperm.

The occurrence of deficiency in the  $F_1$  plants was not determined by means of marker genes or cytological examination in this experiment.



The absence of gross deficiency or translocation could be assumed if all the  $F_1$  plants were free from segregation for defective pollen. It was found, however, that, among about 1000  $F_1$  plants examined, almost 4 per cent showed segregation for defective pollen, ascribable to deficiency or to mutations affecting pollen development. Later studies showed that mutations affecting pollen development are a frequent result of ultraviolet treatment, and that there are also cytologically demonstrable deficiencies induced by the treatment.

No translocations were found among the  $F_1$  plants characterized by defective pollen segregation. In later studies, however, translocations were found, though in very low frequency. The rarity of translocations was given special study because of their very high frequency in comparable X-ray progenies. Since the frequency of deficiency was lower under the ultraviolet doses used than under the X-ray doses commonly applied, it is possible that the difference in effect on translocation is incidental to dosage. If translocations result from chromosome breakage followed by reattachment of broken ends in new combinations, the rarity of translocation following ultraviolet treatment might be due to the smaller number of breaks produced by the ultraviolet dose applied. A further trial was made (Stadler and Sprague, 1937) in which a maximal dose of ultraviolet was compared with a rather low dose of X rays, these doses being approximately equal in total frequency of induced deficiencies for the endosperm genes *A* and *Pr*. The frequency of translocation was determined for each treatment by direct cytological examination of about 100 unselected plants of the  $F_1$  progeny. Only one translocation was found in the ultraviolet progeny, while 44 per cent of the plants of the X-ray progeny showed translocations, several of them two or more independent translocations.

Since it was subsequently found that the frequency of deficiencies in endosperm and embryo is very different under ultraviolet treatment, this comparison represents doses very unequal in frequency of induced deficiencies in the embryos. It therefore does not test the possibility that the rarity of translocations under ultraviolet treatment may be due to the smaller number of chromosome breaks produced. It is clear, however, that, for doses equal in frequency of induced mutation, the frequency of induced translocation is much greater with X rays than with ultraviolet radiation.

Preliminary comparisons of wave-length effectiveness were made in the early maize experiments (Stadler and Sprague, 1936b, c), using the radiation from a commercial mercury-vapor arc with three filters of mercuric chloride solution of varying concentration, and using also the radiation from a commercial mercury discharge tube. The relative effectiveness of the various wave lengths was inferred from the frequency of induced endosperm deficiencies, in terms of the spectral distribution of energy in the filtered radiation applied. The indications regarding wave-length

dependence were quite different from those found in the experiments of Noethling and Stubbe (1936) with monochromatic irradiation of *Antirrhinum* pollen. The results indicated (1) that wave lengths 313  $m\mu$  and longer were relatively ineffective, (2) that wave length 302  $m\mu$  was genetically effective but less effective than wave lengths 297  $m\mu$  and shorter, and (3) that wave length 254  $m\mu$  was much more effective than 297  $m\mu$ . Subsequent studies with monochromatic radiation, reviewed in a later section, confirmed these indications. The maize data are for the frequency of endosperm deficiency, while the *Antirrhinum* data are for the frequency of point mutations. However, tests of mutation rate in the  $F_2$  progenies indicated the same spectral relations for mutations as for endosperm deficiency, as far as could be determined from filtered radiations (Stadler, 1941a).

The frequency of induced embryo abortion was much lower with the longer ultraviolet wave lengths than with the shorter. Comparing doses approximately equal in frequency of induced endosperm deficiencies, the frequency of induced embryo abortion was about nine times as high for the discharge-tube radiation (chiefly wave length 254  $m\mu$ ) as for the filtered radiation (chiefly wave lengths 297  $m\mu$  and longer).

The frequency of endosperm deficiencies induced by ultraviolet treatment of the pollen is very much higher than the frequency of deficiencies affecting the embryos of the same seeds (Stadler, 1941a). Both values can only be estimated, but the discrepancy is too great to be accounted for by any possible error in the estimates.

With maximal doses of the longer wave lengths, the frequency of endosperm deficiencies marked by *A*, *Pr*, and *Su* sometimes exceeds 40 per cent. Since these marker genes can detect only a part of the deficiencies occurring in 3 of the 20 chromosome arms, there are presumably several hundred endosperm deficiencies per hundred seeds. These represent the deficiencies (and perhaps gene mutations) realized under the conditions of endosperm development, from alterations induced in one of the sperm nuclei of the treated pollen grains. The embryos of the same seeds may be checked to provide a maximal estimate of the frequency of deficiencies realized under the conditions of embryo development, from alterations induced in the other sperm nuclei. By this check, every sperm nucleus in the tested population of treated pollen grains may be accounted for. Assuming that every aborted embryo, every plant which failed to yield a pollen specimen, and every plant which showed segregation of defective pollen represents an induced deficiency, the maximum estimate of deficiencies in the gametes tested by embryo constitution is only about 30 per cent.

A similar comparison may be made for endosperm deficiencies and embryo deficiencies marked by specific genes affecting both endosperm and plant characters. Endosperm deficiencies of *A* are very common,



occurring at the doses here considered with frequencies as high as 20 per cent. Deficiency of *A* in the  $F_1$  seedlings is rare; a series of progenies grown from seeds which included 493 endosperm deficiencies for *A* yielded only five *A* deficiencies in the  $F_1$  plants.

Approximately three-fourths of the endosperm deficiencies are fractionals, commonly affecting about half of the endosperm. Deficiencies similarly affecting half of the pro-embryo would presumably be present in the resulting  $F_1$  plants in only about half of the affected cases, and this proportion might be reduced by competitive development of the defective and nondefective sectors. But the proportion of fractionals, as shown by the endosperm deficiencies, cannot account for the discrepancy; among the seeds which yielded the five *A* deficiencies mentioned in the preceding paragraph, there were more than 100 with nonfractional *A* deficiencies in the endosperm.

This pronounced disproportion between the frequency of deficiencies in the endosperm and the embryo does not occur with X-ray treatment. In cultures marked by specific genes affecting both endosperm and plant characters, deficiencies in the  $F_1$  plants are somewhat less frequent than in the endosperms, but not more so than might possibly be accounted for by reduced survival.

Aside from possible differences in the exposure to ultraviolet of the two sperms in the treated pollen grain, a possible cause of the wide disparity is contrasting behavior of chromosome breaks in endosperm and embryo development. McClintock's study (1941) of the effects of mechanically broken chromosomes suggests that some alterations induced in the two sperms, though similar in character and in frequency, might have quite disproportionate effects upon the frequency of detectable changes in the embryos and endosperms of the resulting seeds.

This possibility has been investigated by Schultz (1951), using a ring chromosome (*Dp 3a*) carrying the gene *A<sup>b</sup>*. The pollen treated was that of a *Dp 3a* stock homozygous for a deficiency at the *A* locus, *a-X3*, a type in which all functioning pollen carries the duplication (Stadler and Roman, 1948). Pollinations on ears of *aa* constitution show the loss of *A<sup>b</sup>* in endosperm and embryo tissue by the absence of anthocyanin pigment. With untreated pollen a small percentage of deficiencies occurs in both endosperm and embryo, owing to losses of the ring in late microspore or early endosperm or embryo divisions. The sporadic loss of the ring in subsequent mitoses results in variegation in the remaining endosperms and plants.

The result of a break in the ring chromosome differs from that of a break in a rod chromosome chiefly in the effects of restitution. If the break may be followed alternatively by restoration of the linear order between the original chromatids or crosswise between the sister chromatids, the effect in a rod chromosome would be undetectable in both cases.

But, in the ring chromosome, reproduction must take place in a single plane; otherwise the daughters either will be interlocked or will form a double ring with two centromeres. "Crosswise" restitution in the ring chromosome is therefore likely to result in loss of the ring.

Schultz (1951) found that ultraviolet treatment very greatly increased the frequency of loss of the ring, both in the endosperm and in the embryo. The frequency of deficiencies for  $A^b$  among the  $F_1$  plants was about as high as the frequency of entire (i.e., nonfractional) endosperm deficiencies. This indicates that the low frequency of broken chromosomes found in plants from irradiated pollen, as compared with the endosperm, is largely due to a higher rate of restitution in the plant.

Another interesting result of the study was the absence of any evidence of the production of a rod chromosome from the ring. The type of break which would ordinarily result in a stable terminal deficiency should, if it occurred in a ring chromosome, convert the ring to a rod. This would be detectable as a nondeficient nonvariegated plant. Among more than a thousand seedlings in progenies from ultraviolet-treated pollen, no such plant was found, although the number of  $A^b$  deficiencies presumably induced by the treatments was almost 200. This result is not in conflict with the observed occurrence of terminal deficiencies in other studies. The frequency of deficiencies for a given locus as observed in the  $F_1$  plants is extremely low, and the primary breaks or potential breaks which occur in a rod chromosome proximal to the locus concerned may be much more numerous than those which would occur within the small ring. Presumably the proportion of potential breaks resulting in deficiencies realized in the embryo may be well below one in 200.

The results with the ring chromosome show that the frequency of potential breaks induced by ultraviolet radiation is extraordinarily high, and that in the embryo all but an extremely small proportion of these are followed by restitution. The types of chromosomal alterations found in the deficient plants that make up this small proportion may be quite misleading as to the primary chromosomal effects of the radiation.

The frequency of deficiencies detectable in the endosperm following ultraviolet treatment is comparable with that found following X-ray treatment. The two agents differ widely in the relative frequency of deficiencies affecting the endosperm as a whole and deficiencies affecting only a part of the endosperm ("fractionals"). With ultraviolet, about 75 per cent of the endosperm deficiencies observed are fractionals, and the fraction showing the deficiency is most frequently about one-half of the endosperm, as estimated by the surface area. The deficient sectors vary widely in form and in relative size, sometimes covering only a small fraction of the surface and sometimes covering the entire surface except for a small fraction. The frequency distribution of these fractions of varying size is approximately normal about the modal class of  $\frac{1}{2}$ . Presumably



they represent the distribution of tissue resulting from chance variations in development, when one cell in the two-celled pro-endosperm is deficient.

With X rays, the endosperm deficiencies affect the entire endosperm in most cases. There is a substantial minority of fractionals, but these in general are strikingly different in pattern from those observed with ultraviolet. In most of them, the deficient portion covers the entire surface of the endosperm except for one or more islands of tissue, usually amounting in total to only a small fraction of the entire area. These cases in the X-ray material cannot be regarded as merely the extremes of a range of patterns resulting from random variations in development of the half-deficient endosperm; there is no corresponding frequency of half-and-half mosaics and their variants. The distribution of tissue in the fractionals, as observed in the mature endosperms, suggests that in the ultraviolet cases the nondeficient tissue is the sector derived from one cell of the two-celled pro-endosperm, but in the X-ray cases it is the sector derived from only one or a few cells at a more advanced stage of the pro-endosperm. The distribution of tissue is as would be expected if a chromosome fragment could occasionally escape elimination through one or more cell divisions, and then be restored to normal mitotic distribution.

The elucidation of the breakage-fusion-bridge cycle in mechanically broken chromosomes by McClintock (1941) suggests plausible hypothetical mechanisms by which such endosperm patterns could be produced, though the sequelae of mechanical breakage do not parallel those of either X-ray or ultraviolet alteration of the chromosome.

A comprehensive study of the chromosomal effects of ultraviolet and of X rays, as shown by the mosaic patterns in the maize endosperm, is being made by Fabergé (1951). Using the endosperm marker genes *I*, *Sh*, *Bz*, and *Wx*, all located in a single chromosome arm, a variety of chromosomal effects may be recognized, including rings, dicentric translocations, and inversions, if accompanied by a breakage-fusion-bridge cycle. Ultraviolet treatment produces all these aberrations in large numbers, as does X-ray treatment.

Cytological studies of the chromosomal effects of ultraviolet in maize have been made by Singleton (1939), Singleton and Clark (1940), and De Boer (1945). The accounts of these studies have been published only in abstract form. Singleton (1939) examined  $F_1$  plants identified by the loss of dominant characters present in the treated male parent and found cytologically demonstrable deficiencies of the corresponding chromosome regions in four plants. In another series, cytological examination of  $F_1$  plants identified by segregation for defective pollen showed deficiencies in several cases, including one plant with deficiencies for parts of two chromosomes. All the deficiencies observed appeared to be terminal. No translocations were found. Singleton and Clark (1940) found, among 16  $F_1$  plants with segregation for defective pollen, 8 with observable

deficiencies and 8 without. In addition there was one "deficiency translocation, . . . three-armed, the plant being deficient for parts of chromosomes 1 and 10." De Boer (unpublished) found several similar cases of deficiency and deficiency translocation.

Although all the deficiencies appeared to be terminal, the distinction between terminal and nonterminal deficiencies is not convincing in maize without critical cytological material. Nonhomologous pairing may give a known interstitial deficiency the appearance of a terminal deficiency. De Boer (1945) has presented evidence of ultraviolet-induced terminal deficiency free from this difficulty. The gene *bz* is a plant color gene located on the short arm of chromosome 9, and stocks of maize are available bearing a terminal knob on this arm. Ultraviolet- and X-ray-induced deficiencies of *Bz*, in a stock with the terminal knob, were examined cytologically, the criterion of terminal deficiency being loss of the terminal segment including the knob. Nonhomologous pairing of an interstitial deficiency could result in a terminal unpaired region of the untreated chromosome, but would be recognizable by the presence of the knob, or a portion of the knob, on the deficient chromosome. Among six *Bz* deficiencies in the ultraviolet series, four showed terminal deficiency of the short arm of chromosome 9. The other two were deficiency translocations. Among nine *Bz* deficiencies in the X-ray series, none was a terminal deficiency. These alterations included one interstitial deficiency, three ring-9 configurations, and five deficiency translocations.

Straub (1941), in a study of somatic metaphase chromosomes, has shown that ultraviolet-induced translocations occur with appreciable frequency in *Gasteria*. From some 1800 embryos obtained from the fertilization of untreated eggs by exposed sperm, 210 were selected for cytological study because their weak development suggested that they might possess chromosomal abnormalities. Of these, 75 showed chromosomal changes as contrasted to 1 from 300 control embryos. Four of the embryos were chimeras showing some cells with normal and some with altered chromosomes. Straub considered these to be similar to the fractional endosperm deficiencies induced by ultraviolet in maize. In the remaining 71 embryos, 72 apparently terminal deficiencies were detected in the long arms of the four G chromosomes, which could be recognized by their conspicuous satellites. The breaks giving rise to these deficiencies were largely concentrated in the neighborhood of the centromere. Of the remaining cytological abnormalities, five were translocations, including one of an undefined but probably reciprocal type, two which were defined as "isochromosomes," one dicentric chromosome, and one ring chromosome.

Barton (1954) has compared the chromosomal effects of X-ray and ultraviolet treatments in the tomato (*Lycopersicum esculentum* Mill.). In this plant each of the 12 chromosomes at pachytene shows a densely



staining chromatic region on either side of the chromomere and a lightly staining achromatic region distally. Each chromosome arm terminates in a single well-defined chromomere. This circumstance is very favorable for the detection of terminal deficiencies, especially those in which the break is in the achromatic region.

Barton found a much higher ratio of deficiencies to translocations with ultraviolet than with X rays. The two translocations found with ultraviolet were deficiency translocations. Mutations also showed a much higher ratio to translocations with ultraviolet. The deficiencies found with both ultraviolet and X-ray treatment included both terminal and interstitial deficiencies. Chromosome breakage was highly localized in the chromatic regions; however, the terminal deficiencies observed in the ultraviolet series included one in which the break was in the achromatic region.

The various contrasts in genetic effects which have been mentioned indicate that the chromosome breaks induced by ultraviolet radiation are of a qualitatively different kind from those induced by X rays. Accumulating evidence from many sources has indicated that the mutations induced by X rays are in many cases, if not in all, extragenic alterations incidental to chromosome breakage. The indication that the chromosomal effects of the ultraviolet are of a different kind encourages the hope that the induced mutations also may be qualitatively different.

This possibility may be investigated effectively only by the critical study of the mutation of specific genes, for there are no general criteria by which mutations induced at miscellaneous loci may be distinguished from the possible effects of known extragenic phenomena. With certain selected loci it may be possible to develop special criteria for the identification of gene mutations and for the recognition of alterations which, in experiments on the general mutation rate, would simulate gene mutation.

A comparison of X-ray and ultraviolet mutations of the gene *A* in maize (Stadler and Roman, 1943, 1948) indicates that the mutations induced by the two agents may be qualitatively different. Among about 400 alterations affecting *A*, induced by X rays, and a much smaller number induced by ultraviolet, those most nearly approaching the typical genetic behavior of gene mutations were selected for detailed study and comparison. Among the X-ray alterations, only two were normal plants free from segregating pollen defects. A third haplo-viable X-ray alteration, showing segregating for subnormal but not aborted pollen, was included for comparison. Among the ultraviolet alterations, normal plants with normal pollen were more frequent. Three cases with a phenotype and one with intermediate phenotype ( $A''$ ) were included in the comparisons.

The three X-ray mutants were characterized in varying degree by reduced viability in haplophase and by reduction in the frequency of

crossing over, and were found to be inviable as homozygotes or compounds. These are attributes suggestive of deficiency, and all three mutants were identified as deficiencies by genetic evidence showing that the induced alteration in each case involved loss of the effects not only of *A* but of additional genes affecting chlorophyll development and viability. The four ultraviolet mutants gave no indication of deficiency by any of the criteria mentioned.

The evidence for *Drosophila* comparing the genetic effects of X-ray and ultraviolet radiation contrasts somewhat in its general implications with that from plants. It would not be surprising to find actual contrasts, but the evidence from both sources is still too scanty to force the assumption of any basic difference in the nature of the chromosomal or genic alterations induced. The contrasting indications are briefly noted as follows:

1. Gross chromosomal rearrangements. As shown by Altenburg (1934) and by Muller and Mackenzie (1939), there is no appreciable frequency of gross chromosomal rearrangements in ultraviolet progenies which yield an abundance of sex-linked lethals. Demerec *et al.* (1942) found a single translocation in the progeny of ultraviolet-treated flies, but this single case cannot be considered evidence of an effect of the treatment. The absence of induced translocations in the *Drosophila* cultures tested does not represent a conflict in the evidence, for there is no necessary implication that the radiation is unable to induce translocation in low frequency. The *Drosophila* evidence shows that the ratio of induced translocations to induced sex-linked lethals is far lower with ultraviolet than with X rays. The maize evidence also shows that the ratio of translocations to mutations is far lower than with X rays, and shows further that translocations are induced in very low frequency.

A search for terminal deficiencies of the X chromosome, identifiable by genetic markers, was made by Mackenzie and Muller (1940) and by McQuate (1950), and none was found. The occurrence of ultraviolet-induced terminal deficiencies in maize was shown cytologically by De Boer (1945).

2. Nature of the induced mutations. Although genetic experiments to detect minute rearrangements, by Mackenzie and Muller (1940), indicated that they were absent or very rare in ultraviolet progenies, the direct cytological study of induced lethals by Slizynski (1942) clearly showed that short deletions are included in this class. The frequency of cytologically detectable deletions among the sex-linked lethals was not much lower among the ultraviolet than among the X-ray cases. They occur also, in considerable frequency, among sex-linked lethals arising in untreated material.

The implication from the evidence in maize is that the ultraviolet mutants are distinctly different from the X-ray mutants in that the



former show no evidence of deficiency, while the latter include clearly detectable deficiencies. The evidence of deficiency here is genetic rather than cytological. The only critical evidence is from the induced mutations of *A* studied by Stadler and Roman (1948), and here the number of cases is too small to imply the absence or extreme rarity of short deficiencies among ultraviolet mutations. The fact that no interstitial deficiencies have been found in cytological studies of ultraviolet progenies in maize cannot be considered evidence of their extreme rarity, for here again the only critical evidence, that of De Boer (1945) on *Bz* deficiencies, relates to a relatively small number of cases. It is clear that ultraviolet radiation induces terminal deficiencies in maize, but it is possible that it may induce interstitial deficiencies also, and that some of these may be included among the alterations genetically identified as mutations.

It should be noted also that the alterations identified as mutations in *Drosophila* and in maize may not be analogous classes. The sex-linked lethals spontaneously occurring in *Drosophila* appear to be qualitatively identical with sex-linked lethals induced by X rays, and the ultraviolet lethals are not clearly distinguishable from either class. In maize, the evidence of difference in the type of mutation induced by the two agents comes from studies at a specific locus, at which the X-ray mutants are found to be distinctly different from the spontaneous mutants. Here the ultraviolet mutants are found to be clearly distinct from the X-ray mutants and similar to the spontaneous mutants.

#### DIRECT EFFECTS OF ULTRAVIOLET RADIATION ON CHROMOSOMES

The pollen-tube technique, as employed with *Tradescantia* pollen, permits a study of the direct effects of ultraviolet on chromosomes before inviable aberrations can be eliminated. As discussed earlier, the technique has certain inherent limitations in that only those aberrations realized by the onset of metaphase can be recognized; any which would form at later stages of cell division, or during the process of fertilization, would escape detection. The chromosomes in the generative nucleus are effectively double to both X rays and ultraviolet, and the aberrations induced are consequently of the chromatid types.

In the course of these studies, over 50,000 chromosomes have been examined, and approximately 700 terminal deletions have been identified (Swanson, 1940, 1942, 1943). With the exception of occasional isochromatid deletions or chromatid translocations, which were no more frequent than in untreated nuclei, the aberrations were all terminal. No interstitial deficiencies have been identified with certainty, but it is realized that the method of analysis is such as to preclude their positive identification. The great majority of the deficiencies involved the break-

age of only one of the two sister chromatids, although infrequently half-chromatid deficiencies were observed (Swanson, 1947).

The frequency of terminal deficiencies increases linearly with increasing dosage if the time of exposure after the pollen grains have germinated is

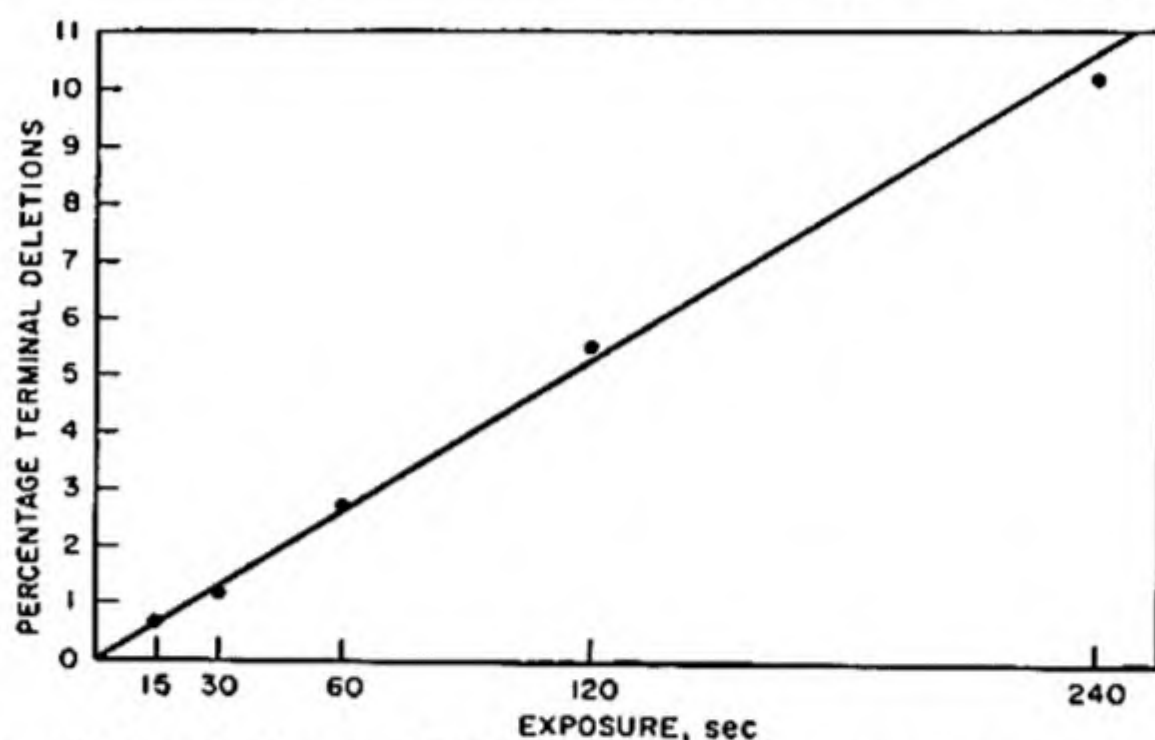


FIG. 7-1. The relation of chromatid deficiencies (terminal deletions) to the dosage of ultraviolet (wave length  $254\text{ m}\mu$ ) in the pollen tube chromosomes of *Tradescantia*. Radiation given at a distance of 20 cm, at an intensity of approximately  $10^3$  ergs/ $\text{mm}^2/60$  sec, and at 2 hr after germination. (Swanson, 1942.)

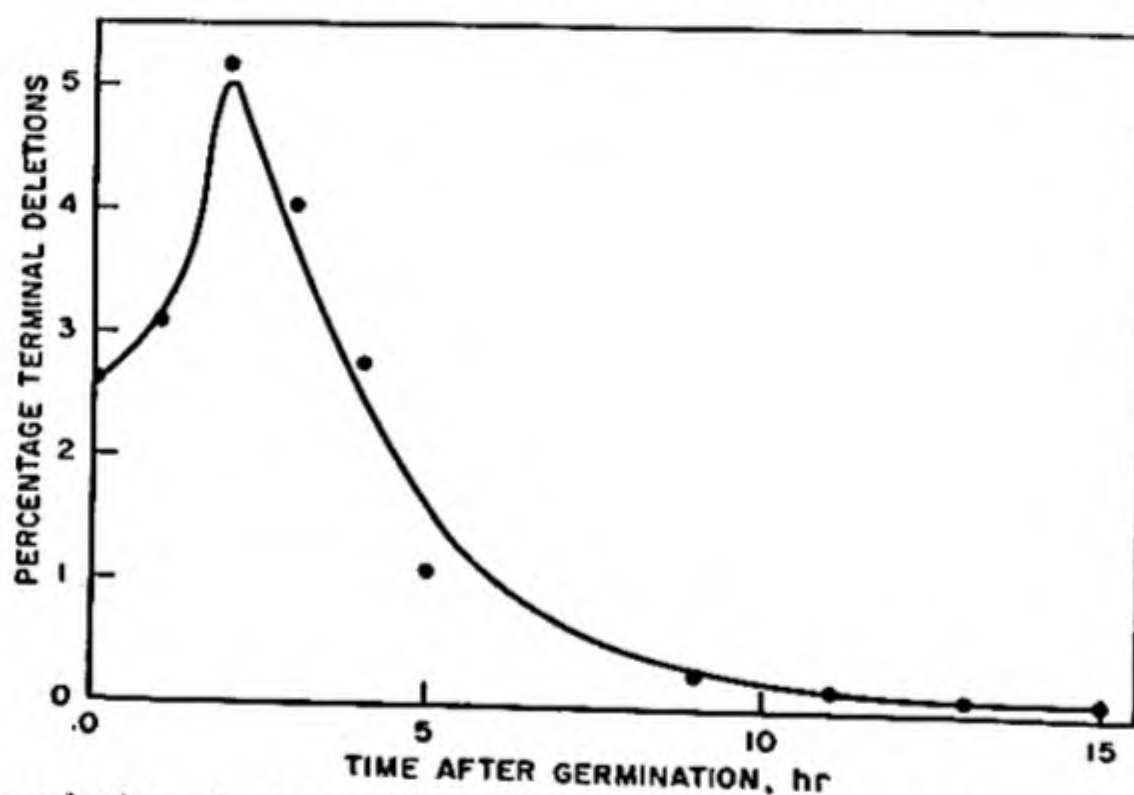


FIG. 7-2. The relation of chromatid deficiencies (terminal deletions) induced by ultraviolet (wave length  $254\text{ m}\mu$ ) in the pollen tube chromosomes of *Tradescantia* to successive prophase stages following germination. Dosage approximates  $2 \times 10^3$  ergs/ $\text{mm}^2/60$  sec. (Swanson, 1943.)

kept constant (Fig. 7-1). With increasing condensation of the chromosomes as they pass through prophase development, the frequency of deficiencies induced by any given dose declines, however, suggesting that internal changes taking place within the chromosome play a role in determining its susceptibility to breakage by ultraviolet (Fig. 7-2). A ques-



tion may be raised concerning the relative resistance of the chromosomes to breakage at the 0- and 1-hr periods. At these time periods the generative nuclei have not, as a rule, passed from the pollen grains into the pollen tubes, and some uncertainty exists as to the degree of absorption of ultraviolet by the heavily pigmented pollen wall. At the 2-hr period, however, the nuclei are in the pollen tube, where absorption by overlying materials is at a minimum. After the 10-hr period the frequency of induced aberrations does not exceed that found in untreated nuclei. This period corresponds roughly to late prophase.

In addition to the terminal deficiencies induced by ultraviolet in the pollen tube chromosomes of *Tradescantia*, there is also found a type of aberration which, for want of a more definite term, has been called an "achromatic lesion" (Swanson, 1940). This type of aberration is induced by X rays also. The lesions extend completely or partially across the chromatid in the form of a nonstainable gap. Their frequency increases with increasing dosage. Whether they represent incompletely separated deficiencies, interstitial losses of chromatin, or merely separated coils within the chromosome is not known. Since many of them extend only a part of the way across the diameter of the chromatid, a large subjective error would be involved in any determination of frequency, and for this reason they have been omitted in the tabulated data.

The nature of the ultraviolet-induced deficiencies in the pollen tube chromosomes of *Tradescantia* suggests that, structurally at least, they are comparable to the fractional endosperm deficiencies in maize even though the changes are induced in dissimilar types of nuclei. Each involves the loss of a portion of a chromatid. No aberrations were found in *Tradescantia*, however, which involved both chromatids, and which would correspond to the entire endosperm deficiencies. Whether this difference can be ascribed to differences in the nuclei studied, to their different states of chromosome condensation, or to some unknown factor cannot be answered at present. Muller (1941) suggests that the preponderance of fractional endosperm deficiencies in maize treated with ultraviolet may result from a mutational process initiated in a single-stranded chromosome but delayed in completion until the chromosome has doubled, the effect being restricted ordinarily to only one of the two chromatids. This hypothesis appears unnecessary in light of the *Tradescantia* data. Since half-chromatid aberrations are found occasionally in treated pollen tube chromosomes, the chromosomes must have at least four strands, and chromatid deficiencies must therefore involve the fracture of two half-chromatids at the same locus. If the chromosomes of the sperm cells of maize pollen have only two strands, the loss of both chromatids by simultaneous breakage to give rise to entire endosperm deficiencies becomes understandable.

Figure 7-3 illustrates the effectiveness of X rays (370 r) in inducing

aberrations in the pollen tube chromosomes of *Tradescantia* at successive prophase periods. A comparison can, therefore, be made with the ultraviolet data obtained under similar circumstances (Fig. 7-2). Isochromatid deficiencies and chromatid translocations are readily induced by X rays, and the close parallelism of the terminal deficiency and translocation curves supports the generally accepted hypothesis that a translocation owes its origin to the illegitimate fusion of the broken ends of two independently induced deficiencies. It would appear that the lack of translocations in the pollen tube following exposure to ultraviolet cannot

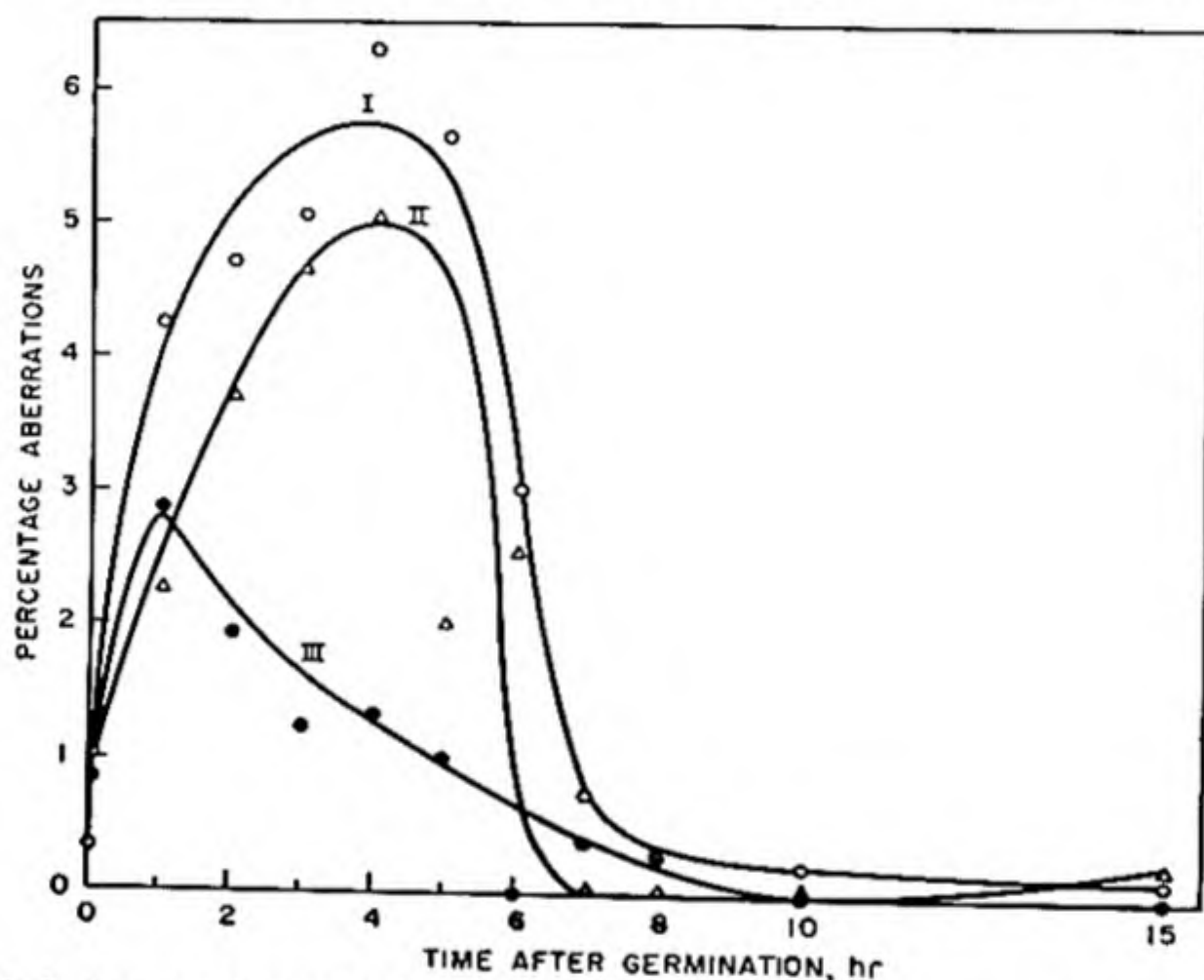


FIG. 7-3. Relation of chromatid aberrations induced by X rays (370 r) in the pollen tube chromosomes of *Tradescantia* to successive prophase stages following germination. Curve I, terminal deficiencies; II, translocations; III, isochromatid deficiencies. (Swanson, 1943.)

be ascribed to a lack of deficiencies. This is more clearly indicated in Table 7-2, where the frequencies of terminal deficiencies induced by the two types of radiation are similar, but those of translocations are not.

The great majority of broken ends of chromosomes induced by ultraviolet clearly do not possess the capacity for subsequent reunion. This may result from a more rapid healing of the broken ends, or it may stem from a lack of maneuverability of broken ends imposed by the surrounding chromosomal matrix. The matrix seems not to be disrupted by ultraviolet, and it may, following heavy doses, actually become more prominent in appearance (Swanson, 1942, 1943).

The terminal deficiencies produced by both radiations in the pollen tube chromosomes of *Tradescantia* are indistinguishable in microscopic appearance, but there is good evidence here, as in maize endosperm, for believing



that they are qualitatively different both as to their nature and as to their mode of origin. When ultraviolet is used in combination with X rays as a pretreatment, the frequency of terminal deficiencies is no greater than that

TABLE 7-2. FREQUENCIES OF CHROMATID ABERRATIONS INDUCED IN THE POLLEN TUBE CHROMOSOMES OF *Tradescantia* BY X RAYS AND ULTRAVIOLET (Swanson, 1942.)

Treatment	Percentage aberrations per chromosome			
	Chromatid deletions	Isochromatid deletions	Translocations	Total chromosomes
Ultraviolet (2537 Å)...	10.15	0	0	936
X ray (123 r).....	10.00	2.7	4.4	1806

expected from the ultraviolet treatment alone (Table 7-3). It would appear that the circumstances which favor the induction of one kind of terminal deficiency actually suppress the appearance of the other kind.

TABLE 7-3. FREQUENCIES OF X-RAY-INDUCED CHROMATID ABERRATIONS AS INFLUENCED BY PRETREATMENT WITH ULTRAVIOLET (Swanson, 1944.)

Treatment	Percentage aberrations per chromosome						Total chromo- somes
	Chromatid deletions		Isochromatid deletions		Translocations		
	Ob- served	Ex- pected	Ob- served	Ex- pected	Ob- served	Ex- pected	
Ultraviolet (2537 Å).....	3.05	.....	.....	.....	.....	.....	3540
X ray (246 r).....	3.51	.....	1.05	.....	1.89	.....	5304
Ultraviolet + X ray...	3.01	6.56	0.35	1.05	0.35	1.89	2256

The X-ray-induced aberrations involved in illegitimate fusion, i.e., isochromatid deficiencies and translocations, are similarly suppressed. Since a like reduction in frequency of X-ray-induced aberrations is encountered when ultraviolet is employed as a posttreatment, it seems reasonable to assume that the action of ultraviolet is not to prevent the initiation of X-ray-induced breaks, but rather to lessen their probability of realization. Kaufmann and Hollaender (1946) have demonstrated that a combination of the two radiations has a similar depressing effect on gross chromosomal aberrations in *Drosophila*, whereas Schultz (1951) has shown that the effects of X rays in maize, as judged by the frequency of germless seeds, are completely inhibited by a posttreatment with wave length 297 mμ at the same time that the ultraviolet effects remain uninfluenced by X rays.

Since the ring-chromosome studies in maize also revealed that breakage by ultraviolet is frequent, a phenomenon obscured in rod chromosomes by restitution, it has been assumed that ultraviolet must have, in addition, a marked influence on the matrices of the chromosomes. Coagulation of the matrices by ultraviolet would thus not only prevent the realization of rearrangements from X-ray-induced breaks but also from those breaks which it itself induces, providing in this manner a mechanism which would lead to an apparent qualitative difference in behavior of the X-ray- and ultraviolet-induced breaks.

### SPECTRAL RELATIONS

Significant comparison of the relative effectiveness of different wave lengths can be made only on the basis of rather precise estimates of the amount of energy reaching the site of the mutagenic reaction. Among higher organisms suited to the genetic analysis of the induced alterations, this is a serious difficulty, and all wave-length comparisons must be interpreted with due regard for the approximations involved in estimating the dose actually applied to the chromosomes whose reactions are determined.

With *Drosophila*, using the technique of irradiation of the adult fly, the comparison of effectiveness of different wave lengths is not feasible. Mackenzie and Muller (1940) estimate that about 99.9 per cent of the ultraviolet energy is absorbed before the radiation reaches the germ plasm. Even slight differences in the relative loss for different wave lengths could make tremendous differences in their relative intensity at the site of genetic action. With the polar cap technique the absorption loss is very much less, but the technical requirements for the identification of the individual mutation make the method unsuitable for experiments on the scale required for wave-length comparisons.

With seed plants adapted to genetic analysis, irradiation of the pollen presents some opportunity for the comparison of wave-length effectiveness. Extensive data on the effects of monochromatic radiations have been reported for *Antirrhinum* and for maize. But even within the single pollen grain, internal filtration results in large and variable differences in the penetration of different wave lengths to the site of the nucleus.

The maize pollen grain is approximately spherical, with a diameter of about 93  $\mu$ . Uber (1939) measured ultraviolet transmission, at different wave lengths, for the pollen grain wall and pollen grain contents of maize. The results indicated that, with equal incident energy at wave lengths 297 and 265  $m\mu$ , for example, the dose penetrating to a point 16  $\mu$  beneath the wall is three times as large for the longer wave length as for the shorter, and at a depth of 32  $\mu$  it is about fifteen times as large. In most of the pollen grains the sperm nuclei are located within this depth range. But,



since the pollen, when treated, is oriented at random with reference to the radiation source, the sperm nuclei in most of the pollen grains treated are at a greater depth, and the filtration losses and inequalities of filtration loss are much greater. Obviously, under such conditions any corrections for dosage must be at best very rough approximations.

In the liverwort *Sphaerocarpus donnellii*, conditions for the comparison of wave-length effectiveness are incomparably better, for the radiation may be applied to the spermatozoid, which is an almost naked nucleus about  $0.5\ \mu$  in thickness. Knapp and Schreiber (1939; see also Knapp *et al.*, 1939) have compared the effects of monochromatic radiations in this organism.

For direct cytological comparison of chromosomal effects, the pollen-tube technique used by Swanson (1940) with *Tradescantia* is also well suited to the study of wave-length effectiveness, with minimal interference from internal filtration of the radiation. Studies of this kind have been exploratory only (Swanson, 1942), but they indicate that considerable wave-length differences are to be expected.

*Dosage Effect.* The comparison of wave-length effects requires a careful consideration of the dosage effect for two reasons: (1) for evaluating the error in approximating equal dosage with the wave lengths compared, because of the varying internal filtration already discussed, and (2) for determining the actual form of the dosage curve, as a basis for interpreting the differences found with the wave lengths compared.

The effect of variation in internal filtration among the individuals treated is to flatten the dosage curve for specific effects. The population treated consists of individuals varying in the proportion of the incident dose that will be received by the gamete nucleus. For example, in a population of maize pollen grains as described earlier, some may be so oriented that the gamete nucleus is reached by radiation that has penetrated through  $16\ \mu$  of overlying material, while in others the gamete nucleus can be reached only by radiation that has penetrated through  $80\ \mu$  of overlying material. The first unit of dosage may produce the effect in any of the pollen grains, and its hits will tend to occur most frequently in the most favorably oriented ones. Added units of dosage may produce additional effects only in the unaffected individuals remaining, which offer a lower probability of hits because of the lowered dose reaching the gamete nucleus in these pollen grains. The flattening of the dosage curve resulting from this factor should occur at all ultraviolet wave lengths, but should be more pronounced at the shorter wave lengths since these show higher absorption in the pollen grain contents. Using observed values for the position of the nuclei within the pollen grain and for the transmission losses in pollen wall and contents, the expected form of the dosage curve at wave lengths 254, 297, and  $302\ m\mu$  was in fairly good agreement with that observed (Stadler and Uber, 1942).

In experiments with the discharge-tube radiation (largely wave length 254 m $\mu$ ), it was possible to show the relation of internal filtration to the dosage curve directly (Table 7-4). A given dose may be applied with

TABLE 7-4. FREQUENCY OF ENDOSPERM DEFICIENCIES FROM A GIVEN DOSE WHEN APPLIED TO BOTH SIDES OF POLLEN AS WELL AS TO ONE SIDE ONLY (WAVE LENGTH 254 m $\mu$ ) (Stadler and Uber, 1942.)

Dose	Frequency, per cent
One unit (one side).....	18.9
Two units (one side).....	24.9
Two units (one from each side).....	35.4

equal effect from either above or below the layer of pollen. When the dose is doubled by applying a second unit of dose from the same direction, the added frequency of induced effects is considerably less than that from the first, and thus the yield from 2 units is considerably less than double the yield from 1. But if, instead, the second unit of dose is applied from the opposite side, the added yield of induced deficiencies is as great as that from the first unit, and thus the yield from 2 units of dose is double the yield from 1.

When the measure of radiation effect is not a specific result (e.g., a given deficiency, death of the irradiated individual) but rather an indefinite group of results, any number of which may be observed in the single treated individual (e.g., mutations at miscellaneous loci), the result expected from internal filtration is not a flattening of the dosage curve. Instead it is a tendency toward coincidence of independent effects in the single treated gamete, such as was noted in the maize experiments mentioned in an earlier section of this review (see also Meyer *et al.*, 1950). A flattening of the dosage curve for mutation frequency would be expected as a result of variations in internal filtration only if the accumulation of mutants and other radiation effects is a factor in eliminating individuals from the population tested.

In treated populations in which there are large variations in exposure to radiation injury, gross distortion of the dosage curve may occur. For example, if the treated population were a mass of pollen grains more than one layer deep, the lower layers would be almost wholly shielded from the radiation. With a sufficiently heavy dose, the pollen grains of the top layer might be largely eliminated from the population tested, and the frequency of genetic effects in the surviving population would be materially lower than that found with lighter doses. The correlation between genetic effects and killing would be a spurious correlation, but any correlation of genetic effects with elimination among the individuals treated would tend to flatten or reverse the dosage curve.

The dosage data for maize pollen treatments are therefore of interest



chiefly in relation to the error involved in wave-length comparisons, rather than to the nature of the reaction of the chromosomes to increasing dose. The results of dosage trials at wave lengths 254, 297, and 302  $m\mu$  are shown in Table 7-5.

TABLE 7-5. RELATION OF DOSE TO FREQUENCY OF ENDOSPERM DEFICIENCY (Stadler and Uber, 1942.)

Dose, $\times 10^3$ ergs/mm <sup>2</sup>	Wave length, $m\mu$	Endosperm deficiencies, per cent $\pm$ S.E.
1.0	254	3.0 $\pm$ 0.6
2.0	254	5.7 $\pm$ 1.1
4.0	254	8.2 $\pm$ 1.6
8.1	254	10.7 $\pm$ 1.3
17.1	254	16.8 $\pm$ 2.6
4.0	297	2.6 $\pm$ 0.7
7.9	297	10.7 $\pm$ 1.1
15.7	297	18.0 $\pm$ 2.2
33.3	297	32.6 $\pm$ 2.3
15.6	302	2.9 $\pm$ 0.7
32.0	302	9.4 $\pm$ 1.0
63.8	302	24.8 $\pm$ 1.8
132.0	302	36.1 $\pm$ 2.7

The relatively low yield of endosperm deficiencies at the lowest doses tested for wave lengths 297 and 302 suggests the possibility of a multiple-hit curve for the dosage relation. This possibility was suggested also by the results of treatments at nine wave lengths in the range 238–293  $m\mu$ , comparing the frequency of endosperm deficiencies from doses of approximately  $0.5 \times 10^3$  ergs/mm<sup>2</sup> with that from doses of approximately  $2 \times 10^3$  ergs/mm<sup>2</sup>. In every case the yield from the lower dose was less than one-fourth of that from the higher dose. Since these treatments were not seriated for the control of daily variation, a separate trial was made with wave length 265  $m\mu$ , using doses of 0.25, 0.50, 1.0, and  $2.0 \times 10^3$  ergs/mm<sup>2</sup>. The frequency of induced endosperm deficiencies was again disproportionately low at the lower doses, but the deviation from linearity was not statistically significant.

Indications of nonlinearity of ultraviolet effect on the frequency of mutations at low doses have also been found in lower organisms. The data of Emmons and Hollaender (1939, Table 2; see also Hollaender and Emmons, 1941) on induced mutation frequencies in *Trichophyton* suggest that low doses are disproportionately less effective than higher doses, although again the departure from linearity is not always obvious or consistent. Similar results have been obtained in *Aspergillus* (Swanson

*et al.*, 1948, Table 2) and *Neurospora* (Hollaender *et al.*, 1945). The statements of Lea (1946) and Catcheside (1948) to the effect that a linear proportionality holds up to the peak of the ultraviolet mutation curve therefore cannot be accepted without question. The need for more precise measurements of mutation effects at low doses is evident, since the interpretation of data on the comparative effects of different wave lengths must depend in part upon the shape of the dosage curve.

The striking drop in mutation frequency observed at very high doses of ultraviolet in fungi (Emmons and Hollaender, 1939) has not been found in maize. This apparently is not related to factors of internal filtration, but rather to the selective elimination of individuals from the population. A somewhat similar drop in frequency has been reported in *Drosophila*. The study by Reuss (1935), in which ventral abdominal exposures of adult males was first employed, provided the first quantitative data on the effects of increasing increments of ultraviolet on the frequency of recessive lethal, semilethal, and visible mutations in *Drosophila*. Exposures of 15, 22.5, and 30 min were used, and a leveling in the dosage relations was evident, but the data are inconclusive because of the small numbers of flies observed. Those obtained by Sell-Beleites and Catsch (1942), following similar methods of exposure, are more striking. In two different experiments (Table 7-6), the mutation frequency rose to a peak

TABLE 7-6. TYPES AND FREQUENCIES OF MUTATIONS IN THE X CHROMOSOME OF *Drosophila melanogaster* INDUCED IN SPERMATOZOA BY VENTRAL EXPOSURE OF THE ABDOMEN TO ULTRAVIOLET RADIATION (Sell-Beleites and Catsch, 1942.)

	Duration of exposure, min	Total no. of flies	Lethals	Visibles	Mutations, per cent $\pm$ S.E.
Expt. I.....	6	2431	26	0	0.92 $\pm$ 0.19
	12	1195	22	1	1.77 $\pm$ 0.38
	24	963	3	0	0.16 $\pm$ 0.13
Expt. II.....	5	2478	8	0	0.17 $\pm$ 0.08
	10	2002	14	1	0.60 $\pm$ 0.17
	15	1443	12	4	0.96 $\pm$ 0.26
	20	1464	6	0	0.26 $\pm$ 0.13

with increasing increments of ultraviolet and then dropped abruptly as added increments were given.

Sell-Beleites and Catsch concluded that they were dealing with a greatly disturbed "one-hit" curve, and that the decrease in mutation frequency at high doses results from increasing sterility induced by the penetrating radiation. Many of the irradiated flies were completely sterile or yielded no mutations of any kind. The percentage of sterility



increased with increasing dosage and resulted presumably from physiological damage unassociated with the mutation process. Once a maximum mutagenic effect was obtained in those individuals in which the radiation had successfully penetrated, added increments of ultraviolet would be likely to contribute more rapidly to sterility than to the induction of additional mutations. A drop in mutation frequency would therefore be expected since the fertile flies with a high degree of filtration and a low frequency of mutations would contribute a disproportionately greater number of offspring to the next generation.

Attempts to determine dosage relation in *Drosophila* by the polar cap technique have been reported. Altenburg *et al.* (1950) indicated that a linearity of effect obtains at low doses, but that a leveling of the curve is rapidly achieved, after which large increments of dose are relatively ineffective in raising the frequency of mutation. Meyer *et al.* (1950) also found that a clustering of mutations occurs in cells or chromosomes favorably oriented with respect to the radiation. A study of 11 chromosome II lethals showed that 10 of these chromosomes carried additional mutations. Also, increases in dosage caused death to an increasing number of pole cells, as evidenced by the greater proportion of  $F_1$  offspring carrying a particular mutation.

Perhaps the best evidence on the relation of ultraviolet effects to dosage, from the standpoint of the avoidance of internal filtration difficulties, is that from *Tradescantia* pollen-tube treatments. Chromatid deficiencies here provide the criterion of effect (Swanson, 1942). The data indicate that the relation is linear. Figure 7-1 illustrates the curve obtained with wave length  $254\text{ m}\mu$ .

*Wave-length Dependence Studies.* The earliest studies of the differential genetic effectiveness of various wave lengths of monochromatic light on the germinal material of higher plants were made by Noethling and Stubbe (1934, 1936; see also Stubbe and Noethling, 1936). The dry pollen of *Antirrhinum majus* was treated, and the detection of induced mutations was made by observing the segregations in  $F_2$  populations. The control populations had a comparatively high frequency of spontaneous mutations (1.6 per cent). From a study of  $F_2$  offspring from some 3000 pollen grains treated with wave lengths 265, 297, 302, and  $313\text{ m}\mu$ , the authors were able to show that each wave length was capable of increasing significantly the frequency of mutations, the highest rate obtained being approximately four times the control frequency.

Several doses were compared at wave lengths 265 and  $297\text{ m}\mu$ . At wave length  $265\text{ m}\mu$ , doses of  $2 \times 10^3\text{ ergs/mm}^2$  failed to raise the mutation frequency significantly, and the rise with increasing doses was slow. At doses of  $2.7 \times 10^3\text{ ergs/mm}^2$ , the frequency was twice that of the controls, while doses of  $14.6 \times 10^3$  and  $50.3 \times 10^3\text{ ergs/mm}^2$  increased it only to three times that of the control. The use of wave length  $297\text{ m}\mu$

in a wide range of doses ( $1.8 \times 10^3$ – $65.0 \times 10^3$  ergs/mm<sup>2</sup>), gave significant increases over the control, but no significant differences between doses. A dose of  $65 \times 10^3$  ergs/mm<sup>2</sup> at wave length 302 m $\mu$  and one of  $120 \times 10^3$  ergs/mm<sup>2</sup> at wave length 313 m $\mu$  quadrupled the spontaneous frequency. From these results the conclusion was drawn that the peak of genetic effectiveness was in the neighborhood of 300 m $\mu$ . Supporting this hypothesis was the demonstration that a suspension of *Antirrhinum* pollen in 30 per cent alcohol had a maximum of absorption around 300 m $\mu$ .

Comparable data by Stadler and Uber (1938) for the frequency of endosperm deficiencies induced by monochromatic radiations also showed effects in large numbers for all wave lengths tested in the range 235–302 m $\mu$ . Longer wave lengths had no appreciable effect. The frequency of germless seeds was increased markedly by radiation of wave lengths 280 m $\mu$  and shorter, but not by longer wave lengths, even at much higher doses. The tabular data on which these conclusions were based were later published (Stadler and Uber, 1942, Table I), together with the results of additional experiments on relative wave-length effectiveness.

The frequency of induced effects was higher for wave length 297 m $\mu$  than for 265 m $\mu$ , as in the experiments of Noethling and Stubbe. But, though wave length 297 m $\mu$  significantly excelled 265 m $\mu$  at the higher doses, the relation was clearly reversed at the lower doses. Both wave lengths were tested at doses of approximately 1, 2, 3, 6, and  $12 \times 10^3$  ergs/mm<sup>2</sup>. While the longer wave length was about twice as effective as the shorter in comparisons made at the heaviest dose, it showed no effect at the lowest dose, and only about one-fourth of the wave length 265 m $\mu$  effect at the second dose. The results as a whole showed that there were wide differences in the dosage relations at the different wave lengths tested, and that a study of the dosage relation and of internal filtration was needed before the relative effectiveness of the wave lengths could be estimated. The results of these studies were summarized in the preceding section.

The most reliable indications of relative effectiveness come from comparisons made at low doses. However, at the lowest doses filtration losses are still effective and require correction. The frequency of endosperm deficiencies produced in an experimental comparison of seven wave lengths, all at a dose of  $2 \times 10^3$  ergs/mm<sup>2</sup>, seriated for the control of daily fluctuations, is shown in Table 7-7.

At this dose, the endosperm deficiencies induced by wave lengths 265 and 254 m $\mu$  are about 3 times as frequent as those induced by wave length 297 m $\mu$  and about 15 times as frequent as those induced by wave length 302 m $\mu$ . When the dose is increased to about  $8 \times 10^3$  ergs/mm<sup>2</sup>, the differences are much less pronounced and in some cases hardly significant. The evidence on internal filtration, subject to the approximations which have been mentioned, indicates that wave lengths 265 and 254 m $\mu$



are probably more than 10 times as effective as wave length 297 mμ and probably more than 100 times as effective as wave length 302 mμ, in terms of equal intensity at the surface of the sperm nucleus.

TABLE 7-7. THE FREQUENCY OF ENDOSPERM DEFICIENCIES IN MAIZE AS A FUNCTION OF WAVE LENGTH AT TWO DIFFERENT DOSES (Stadler and Uber, 1942.)

Wave length, mμ	Endosperm deficiencies, per cent ± S.E.	
	At $2 \times 10^3$ ergs/mm <sup>2</sup>	At $8 \times 10^3$ ergs/mm <sup>2</sup>
248	9.9 ± 1.0	22.9 ± 2.3
254	15.5 ± 1.3	23.2 ± 2.2
265	11.6 ± 1.0	15.2 ± 1.2
270	7.8 ± 1.6	20.9 ± 1.9
280	9.3 ± 0.9	23.7 ± 1.5
289	6.2 ± 0.8	18.5 ± 1.5
297	4.7 ± 0.5	11.4 ± 1.2

In the comparison of wave-length effects in *Sphaerocarpus*, complication from internal filtration is at a minimum, for the spermatozoid irradiated consists almost entirely of nuclear material. Knapp *et al.* (1939) compared the effectiveness of six wave lengths in inducing genetic alterations in *Sphaerocarpus donnellii*. The spermatozoids were irradiated in water suspension, and the sporogonia produced by fertilization of untreated

TABLE 7-8. THE FREQUENCY OF INDUCED MUTATIONS IN *Sphaerocarpus* AS A FUNCTION OF WAVE LENGTH, DOSAGE  $2 \times 10^3$  ERGS/MM<sup>2</sup> (From Knapp *et al.*, 1939.)

Wave length mμ	No. of sporogonia analyzed	No. of mutations	Percentage mutations
254	61	17	27.8
265	53	22	41.8
280	64	14	21.9
297	52	3	5.8
302	71	4	5.6
313	65	0	0.0
Control.....	64	0	0.0

female gametophytes were tested by analysis of the spore tetrads. Mendelizing mutations were identified by the production of two normal and two mutant plants; lethal "mutations" (i.e., all genetic alterations with haplo-lethal effect), by the production of only two instead of four plants by the spore tetrad. By the tetrad analysis of 50 to 75 sporogonia representing each treatment, it was possible to show sharp differences in

the effectiveness of some of the wave lengths applied in inducing such alterations. Radiation injury was in general parallel to genetic effectiveness. The data are shown in Table 7-8.

These data represent the most significant evidence now at hand, bearing upon the action spectrum of the ultraviolet in producing genetic effects. It is interesting to note that wave length 265  $m\mu$  is about seven times as effective as wave lengths 297 and 302  $m\mu$ , and that the two latter wave lengths are not appreciably different in effectiveness. The effectiveness of wave length 254  $m\mu$  is about two-thirds that of 265  $m\mu$  though the significance of this difference is perhaps questionable. The effectiveness of wave length 280  $m\mu$  is about half that of 265  $m\mu$ . From the resemblance between these values and the absorption spectrum of nucleic acid, the authors conclude that absorption by nucleic acid is of essential significance in determining the genetic effects of ultraviolet radiation.

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## CHAPTER 8

# The Effects of Radiation on Protozoa and the Eggs of Invertebrates Other than Insects\*

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*Introduction. Lethal effects: Kind of death—Sensitivity—Recovery—Substances present in the medium during irradiation—Effect on the medium. Retardation of cell division: Recovery—Sensitivity—Localization. Inherited effects. Miscellaneous effects: Activation of eggs—Excystment of protozoa—Motility and behavior of protozoa—Sensitization to heat—Miscellaneous microscopically visible changes—Various physiological, biophysical, and biochemical effects. References.*

## INTRODUCTION

From the point of view of a biologist interested in the effects of radiation there is a certain unity between investigations with protozoa and with the eggs of marine invertebrates—in both instances, one is dealing with single cells in liquid medium; in both, the methods employed have tended to emphasize the cell as an individual rather than as a member of a population. So, while the connection between investigations with the two kinds of material has sometimes been slight, it seems proper to consider them together.

The review could have been organized in many ways. The actual choice, a consideration of each kind of effect separately, declares the point of view to be that of an experimental biologist rather than a biochemist or biophysicist. It seems important to stress that such frequently employed radiobiological criteria as death and division delay may not be the same in all materials. Actual occurrence of death of cells may be observed, not just the end result, such as failure to produce daughter cells in large numbers. Division delay may be examined in detail rather than as an overall effect on the number of cells or mitoses in a population. From such studies, it is clear that a variety of phenomena are concerned. When investigations on many organisms have been made, it may be possible to recognize certain phenomena which are common to all. Until this is

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done, any attempt to treat death, division delay, etc., as having the same cause in all cells is likely to be misleading.

This review has been limited, for the most part, to papers appearing from the year 1935 through the year 1950. A few earlier pieces of work have been included when they seemed especially important for the topics discussed, or when they were early papers in a series which continued beyond 1935. A number of new lines of investigation have developed since this manuscript was submitted. It has not been feasible to revise the manuscript to include them. References to most of this work may be found in Giese (1953), Wichterman (1953), and in Nuclear Science Abstracts.

### LETHAL EFFECTS

*Kind of Death.* Many investigations have been concerned with the lethal effects of radiation. However, the nature of the death is certainly not the same in different cases. A variety of possible causes of death will be discussed in the following paragraphs.

First, there is death which occurs during or shortly after irradiation. Numerous investigators have observed such death and described in more or less detail the accompanying phenomena, such as loss of motility, vacuolization and coagulation of the cytoplasm, and other changes. At least a part of the investigations recorded in Tables 8-2 and 3 involve phenomena preliminary to death.

This type of death may occur very rapidly, as shown by the observations of Rentschler and Giese (1941) and Harvey (1942), with intense flashes of ultraviolet. Some organisms, notably the ciliates, disintegrate within a few seconds. In other cases, some minutes or even an hour or so may elapse between irradiation and disintegration.

The doses of ionizing radiation required to bring about this type of death are very high. Dognon and Piffault (1931a) estimate the immediate lethal dose for *Paramecium* to be about 500,000 r. Halberstaedter (1938) showed that a dose of more than 100,000 r of X rays was needed to immobilize *Trypanosoma gambiense*. Halberstaedter and Back (1942) report loss of motility and cytolysis of the colonial flagellate *Pandorina morum* after doses of 300,000–600,000 r. The very high doses required for immediate killing have sometimes been considered characteristic of the protozoa. However, Scott (1937) in his review points out that immediate, as contrasted to delayed, death requires doses of similar magnitude in other organisms.

Giese and Leighton (1935a) report on the immobilization and vesiculation of *Paramecium* by monochromatic ultraviolet. In both cases 2804 Å appears to be more effective than other wave lengths. However, calculations of quantum efficiency, based on measurement of absorption of paramecia, suggest that 2654, 2804, and 3025 Å were about equally effec-

tive, whereas 2537 Å was less effective. McAulay and Taylor (1939) investigated the death (by bursting) of *Paramecium* as a result of exposure to monochromatic ultraviolet. They found that all wave lengths up to 3000 Å were quite effective but that effectiveness was much less at longer wave lengths. It may be doubted that their techniques were sufficient to detect differences in effectiveness among the shorter wave lengths. Shettles (1938), using the flagellate *Peranema trichophorum*, observed death after 2 hr exposure to 2537 Å ultraviolet but no death after 8.5 hr exposure to 2650 Å. Likewise, no death was found after comparable exposures to longer wave lengths. The intensities of the different wave lengths are said to have been equalized by use of a photometer. Thus no general conclusions are possible about wave-length dependence of immediate death after ultraviolet.

It is probable that immediate death is due to extensive damage to the cellular material. This is suggested by both the rapidity with which it can occur and the very large doses of ionizing radiation necessary to bring it about. It might be surmised from the microscopic observations that coagulation of the protoplasm or damage to the cell membrane, or perhaps both, are involved, but no really conclusive evidence is available. Possibly, different kinds of damage lead to death in different cells under different circumstances of irradiation.

It is rather generally accepted (Lea, 1947) that irradiated cells may be able to survive until they divide, at which time death is caused by loss of parts of chromosomes as a result of chromosome aberrations induced by the radiation. Such a mechanism is suggested by Holweck and Lacasagne's observation (1931a, b) of death at division in the flagellate *Polytoma*. At certain doses of  $\alpha$  particles, many of the cells remained normal until division and then disintegrated. Using the hit theory, they calculate that a two-particle event is involved with a target diameter of 2.3  $\mu$ , corresponding roughly to the area presented to the particles by the pericaryosomal space in the nucleus, the region in which, presumably, the chromosomes are located. Similarly, Halberstaedter and Back (1942) report that *Pandorina* colonies exposed to between 3000 and 300,000 r remain normal until cell division, when cytolysis takes place. This was so regardless of whether old colonies that underwent cell division shortly after irradiation or young colonies that did not undergo cell division for some time were used.

When the test of survival is the ability of the cell to multiply and produce a culture, death due to mutations or chromosome aberrations must be considered as a possible explanation, although other causes cannot be excluded. Two cases showing the ability of free-living flagellates to produce cultures after X irradiation may be mentioned. Ralston (1939) distinguishes immediate from delayed death and reports the median lethal dose for delayed death in *Dunaliella salina* as between 10,000 and 13,000 r.



Schoenborn (1949) reports the median lethal dose for *Astasia longa* to be between 20,000 and 40,000 r.

There have been a number of investigations of the killing of parasitic protozoa by radiation; and in most of these the criterion was the ability to infect the host after irradiation *in vitro*. Thus chromosomal changes may have been involved. Haberstaedter (1938) found that a dose of 12,000 r of X rays to *Trypanosoma gambiense*, *in vitro*, was sufficient to prevent the infection of mice, whereas doses of more than 100,000 r were necessary to produce any obvious changes in the motility of the trypanosomes. Other investigations with parasitic protozoa are summarized in Table 8-1.

There are a few cases of delayed death in which a genetic explanation is more difficult. Holweck and Lacassagne's case (1931a, b) with *Polytoma* in which death occurred after division has already been mentioned, and the interpretation has been suggested that chromosomal aberrations were involved. Using  $\alpha$  particles, they distinguished other kinds of effects as follows: (1) the cells remain motile and grow in size but finally lyse without division; (2) the cells are immobilized but grow, yet they finally lyse without division; (3) the cells become immobile, fail to grow, and lyse without division. These workers (see also Lacassagne, 1934a, b; Holweck, 1934) made sensitive volume calculations for the first and the last two effects and found for the first a volume approximately the equivalent of the centrosome, whereas for the last two, the volume was approximately that of the kinetosomes. These rather remarkable identifications presumably would offer an explanation for the failure to divide and for the immobilization, but would not in themselves account for the eventual death. Holweck and Lacassagne (1931b) suggest that death is due to the suppression of reproduction and of motility. However, *Polytoma* cells are probably haploid and so lethal mutational changes could be invoked as an explanation on the assumption that a mutation can express itself in the cell in which it arises. Nevertheless, the possibility must be borne in mind that this is a case of delayed death due to causes other than mutational change.

In the ciliate protozoan, *Paramecium*, a nongenetic explanation for delayed death appears by far the most likely one. Sonneborn (1947) has shown that the macronucleus in this ciliate contains many sets of genes. It is therefore improbable that loss of chromosomal material or gene mutation in either the macronucleus or micronucleus would have much effect on ciliates multiplying vegetatively. In order to allow for such an effect the unlikely assumption would have to be made that a given mutation or deficient chromosome is dominant over many normal genes or chromosomes. Alternatively, the radiation would have to be assumed to cause sufficiently extensive damage to the chromosomes to lead to death even with many sets.

For this reason, *Paramecium* and other ciliates might be expected to be

relatively well buffered against radiation damage and, in a sense, this is true. The continued reproduction of the flagellates is prevented by a few tens of thousands of roentgens of X rays, but hundreds of thousands are needed to prevent the vegetative multiplication of ciliates. Back (1939) found for *Paramecium caudatum* that a dose about two-thirds the immediate lethal dose, i.e., about 300,000 r, led to eventual death. The animals survived for several weeks but decreased in size, and finally died. Lacassagne (1934b), working with *Glaucoma scintillans*, had previously reported death after several days without division. However, he found that some increase in size occurred. Thus delayed death does occur in these organisms but it occurs without division.

Kimball and Gaither (1951) have been able to distinguish at least two kinds of delayed death in *Paramecium aurelia* following exposure to ultraviolet. With higher doses, some of the animals survive a day or more without division, but eventually die. Long periods of the kind reported by Back (1939) for X rays were not observed for death without any division. However, at slightly lower doses, the animals pass through two or three divisions rather slowly (in 2 or 3 days) but then cease dividing entirely for several days. Some animals finally recover the normal division rate after remaining undivided for periods as long as three weeks, but others die during this "cessation" period. All animals, whether they eventually recover or die, become very small and thin during this cessation of division, and apparently all come very near death. From the decrease in size of the animals, it seems probable that the delayed death of *Paramecium* involves an effect on the synthetic processes within the cell. Death could well result when the cell, no longer capable of making new material, comes to the end of its resources. In the case of ultraviolet, several divisions ordinarily elapse before the synthetic processes come to a halt, while with X rays, Back's study (1939) suggests that this is not so. Also, recovery has been found for the ultraviolet effect but no recovery has been reported for X rays. Whether these differences are real or only apparent remains for future investigation. In any case, it seems hardly possible that death during the cessation period after ultraviolet irradiation is due to mutational changes, since recovery can occur even in the sister animals of those which die. It also seems improbable that enough genetic damage could be done either by X rays or ultraviolet to cause the delayed death without division, although perhaps such an explanation is just possible with the large doses of X rays employed. Nonetheless, death due to mutational changes is known for *Paramecium aurelia*. Animals given doses of 10,000 r or less divide normally as long as they multiply vegetatively, but when they undergo the self-fertilization process of autogamy many of the exautogamous clones are inviable. This phenomenon will be discussed in more detail in the section on inherited effects.



The killing of nematode eggs by radiation may involve, in addition to death of cells, rather diverse processes, such as abnormal development of embryos and failure of the young worms to hatch. Several investigations with ultraviolet will be reviewed here. The eggs of *Ascaris* have been, in the past, favorite subjects for radiobiological research with X rays but most of this work can be found summarized in Duggar (1936) and in the tables of Dognon and Biancani (1948).

Wright and McAlister (1934) examined eggs of *Toxocara canis* and *Toxascaris leonina* for embryonation after exposure to 3650, 3130, 3022, 2967, 2804, and 2650 Å monochromatic ultraviolet. At the lowest dose used (6840 ergs/mm<sup>2</sup>) effects were found only with the two shortest wave lengths. Effects were found with 3022 Å at a dose of 274,000 ergs/mm<sup>2</sup>, but even 1,370,000 ergs/mm<sup>2</sup> of the two longest wave lengths had no effect.

These results are in agreement with those of Jones and Hollaender (1944) with *Ascaris lumbricoides*. They found that energies of about 6,000,000–8,000,000 ergs/mm<sup>2</sup> of ultraviolet of the wave-length band 3500–4900 Å were needed to prevent embryonation of a large part of the eggs. Slightly higher energies were needed to prevent the hatching of the eggs of the pinworm *Enterobius vermicularis*. These very long wave lengths do have a lethal effect but only at very high doses. In an earlier communication Hollaender *et al.* (1940) show that the action spectrum for the prevention of hatching of *Enterobius* eggs has a small peak at 2804 Å and then rises rapidly to the shortest wave length tested, 2280 Å. This action spectrum is similar to the absorption spectra of some proteins and lipids. Hollaender and coworkers suggest, as possible modes of action, hardening of an outside protein layer of the shell, change in composition of the lipoid membrane, damage to the embryo, or production of toxic substances within the egg. The last is considered unlikely at the energies used.

In summarizing this section on kinds of death, it would appear that at least the following categories of cell death can be recognized: (1) death within a maximum of a few hours after irradiation, (2) death after considerable periods of time but without division, (3) death at or shortly after the first division, (4) death after several divisions, and (5) death following sexual processes. It seems quite possible that both (3) and (5) are the result of gene mutations and chromosomal aberrations and so it is not surprising to find that they occur in detectable amounts even at quite low doses. The very high doses characteristic for (1), together with the immediate changes involved, suggest extensive damage to cellular materials. The death in both (2) and (4) may involve disturbances in the synthetic processes of the cell which finally lead to death when the resources of the cell are exhausted. In some instances, mutational changes can be excluded as a probable explanation of (2) and (4). When

the ability of the cells to produce cultures, or infections in the case of parasitic forms, is investigated, death will be due, obviously, to the most sensitive of these processes occurring within the part of the life cycle investigated.

*Sensitivity.* The sensitivity of individual cells of the same species to the lethal effects of radiation varies considerably. Halberstaedter and Back (1942) found that all cells in a colony of *Pandorina* died at the same dose although different colonies required different doses. Secondly, they found, by giving repeated increments of 100,000 r and examining for immediate death after each increment, that the colonies of a small clone (16 colonies) all died at the same, or approximately the same dose; but colonies of different clones died at quite different doses. In this way some clones were found which died at 300,000 r and others which died at 600,000 r.

Back and Halberstaedter (1945) demonstrated a somewhat similar phenomenon with *Paramecium caudatum*. When groups of about eight paramecia from cultures derived from a single animal by at least six divisions were tested, it was found that the eight might die at quite different doses. However, the four to eight animals derived from one, by two or three divisions, were found to die almost invariably at the same dose, although different groups died at quite different doses. Therefore, the sensitivity is the same for closely related individuals but not for the more distantly related ones.

This investigation of Back and Halberstaedter is of much interest, for it suggests that minor variations between cells may lead to rather marked changes in sensitivity. The evidence also suggests that these minor variations may be maintained over a few cell divisions. It is improbable that the similarity between products of a single paramecium could be due to the products being in the same part of the fission cycle, for the numbers given in the tables suggest that Back and Halberstaedter (1945) often used groups in which some of the animals had divided once more than the others.

Different stages in the life cycle of a given species may be of different sensitivity. Tang and Gaw (1937) find that older cultures of *Paramecium bursaria* are more susceptible to the immediate lethal effects of ultraviolet than younger ones. Brown *et al.* (1933) report that cysts of the ciliate *Euplotes taylori* are killed by approximately 400,000 r of X rays, whereas the motile form requires approximately 460,000 r. The criterion in this case was the ability to survive 48 hr. However, they suggest that this difference may have been due to the different media in which the cysts and motile forms were kept. Bennison and Coatney (1945) found that 8000 r of X rays prevented infection of chicks by a suspension of sporozoites of *Plasmodium gallinaceum*, while 20,000 r was required to prevent infections by suspensions of trophozoites. Here, as in the work with



*Euplotes*, the possibility of an effect of the medium during irradiation must be considered. Packard (1924) reported what appears to have been a major difference in sensitivity to radium (principal effect said to have been due to slow  $\beta$  particles) between two species of ciliates. The lethal dose for *Paramecium* was 3 hr exposure to the source while that for the hypotrichous ciliate *Stylonychia* was 15 hr exposure. Since *Paramecium* is very resistant to ionizing radiation, this result is quite surprising. Packard attributes the difference to the lesser permeability of *Stylonychia*. Unfortunately, there has been no other work with *Stylonychia*; however, the related hypotrich, *Euplotes*, is killed at approximately the same dose as *Paramecium* (Brown *et al.*, 1933). More recently, Wichterman (1948a, b) has reported results which suggest a small difference in susceptibility to X rays between *Paramecium bursaria* and *P. calkinsi*. He has stated that some *P. bursaria* survived doses between 400,000 and 600,000 r, whereas all *P. calkinsi* were killed by 400,000 r.

More striking differences have been reported in the lethal doses of ultraviolet. Rather extensive comparisons between different species and strains of protozoa, primarily ciliates, are to be found in the works of Giese and Leighton (1953b) for long wave lengths, of Giese (1938b) for shorter wave lengths, and of Harvey (1942) for intense flashes of ultraviolet. Giese (1946b) has made similar comparisons for the eggs and sperm of a number of marine invertebrates. Shalimov (1935) reported that eggs of *Ascaris equorum* and *Enterobius vermicularis* were killed by ultraviolet in 5 min, whereas those of *Strongylus equinus* were killed in only 3 min. Wright and McAlister (1934) found *Toxascaris* eggs to be more readily affected by monochromatic light than were the eggs of *Toxocara*. They suggest that this may be due to differences in the absorption of the shell. This emphasizes the difficulties which are inherent in interpreting differences in sensitivity to ultraviolet. Differences in absorption in the outer layers of cytoplasm or egg shells and differences in the action spectrum for superficially similar effects may be involved, as well as more subtle differences in the biological organization of the organisms being compared.

*Recovery.* The possibility that recovery may occur from changes which ordinarily lead to death has been investigated by use of fractionated doses. The method assumes that the effect is not due to a single "hit." If this is true and recovery does occur, then fractionated doses with sufficiently long rest periods in between the fractions should have less effect than the same total dose given as a single exposure in a brief period of time. Such a decreased effectiveness of fractionated doses of X rays has been reported by Crowther (1926) for the ciliate *Colpidium colpoda* and by Back (1939) for *Paramecium caudatum*. Crowther (1926) found that one dose given in about 20 min produced death, whereas a dose one and a half times greater was required when given as three exposures at 2-hr intervals. It

can be estimated from Crowther's data that the doses used were of the order of  $10^5$  r. Back (1939) reported that about half the immediate lethal dose given every day for 3 to 4 successive days produced the same effect as two-thirds the immediate lethal dose given in a single exposure; i.e., the paramecia survived for some time without division, but eventually died. Since the final dose was several times the single exposure dose for immediate death, it can be concluded that fractionated exposures were less effective than single exposures. Quite different results were reported by Berner (1942) for immediate killing of *Paramecium caudatum*. He found that doses of X rays given in fractions, one fraction every 24 to 48 hr, were considerably more effective than doses given at more frequent intervals. He presented evidence that X irradiation decreased the mineral content as shown by ashed preparations and that recovery from this decrease was just complete in 48 hr. He believed that at this time the animals were more susceptible to X rays because their reserves had been depleted by the recovery process. The results of his investigations with ashed preparations and with death were quite variable, indicating a need for further investigation before these conclusions can be fully established. Nonetheless, the idea that a recovery process may lead to a temporary increase in sensitivity to radiation is an important one.

Halberstaedter and Back (1942) found that fractionation into several parts with 1 or more days between had no noticeable effect on the action of X rays in *Pandorina morum*, either on immediate death or death after division. Halberstaedter and Luntz (1929) had previously found a similar lack of effect of fractionation of the dose of radium rays on the related species *Eudorina elegans*. As just pointed out, death after division in *Pandorina* might be due to chromosomal aberrations. If a large proportion of the total were attributable to one-hit aberrations, the failure to find an effect of fractionation would be expected. However, the lack of an effect of fractionation on immediate death is surprising, especially since doses of 300,000 r and greater are necessary to bring it about. On the other hand, Forssberg (1933) found a marked effect of the intensity of X rays on killing and division delay in the single-cell algae, *Chlorella vulgaris*, *Scenedesmus basiliensis*, and *Mesotaenium caldariorum*. The effectiveness increased with intensity, and reached a maximum at about 1600 r/min. Halberstaedter and Back (1942) used intensities of 9000 r/min and greater. Thus it is possible that this intensity was too high to allow discovery of an effect of fractionation.

*Substances Present in the Medium during Irradiation.* There have been a number of reports of the combined action of radiation and substances of one sort or another added to the medium. Dognon and Piffault (1931c) reported that the lethal dose of X rays for *Paramecium* was distinctly decreased in the presence of several dyes and toxic salts, e.g., potassium cyanide or iodide. Preliminary irradiation of the compounds had no



effect but irradiated paramecia added to the compounds died more rapidly than the controls. Resorcinol and sodium hyposulfite protected against the combined action of radiation and these compounds. These authors believed that the death was due to the easier penetration of toxic substances brought about by a radiation-induced increase in the permeability of the membrane. Black (1936) has studied the effects of a number of salts on the cytolysis of *Amoeba proteus* by ultraviolet irradiation. Koehring (1940) has shown that the ameba, *Chaos chaos*, is more readily killed by a combination of neutral red and the radiation from radon than by either alone. Bohn (1941) found that paramecia in various salt solutions, dyes, etc., survived quite normally in the dark but were killed in a few hours on exposure to visible light. It is possible to consider all these effects as due to increased permeability to injurious substances, but it should be emphasized that there is no complete agreement that permeability is changed by irradiation (see Table 8-4).

Levin and Piffault (1934a, b, c) have found that *Paramecium aurelia* placed in suspensions of lecithin or of cholesterol become resistant to the immediate killing action of X rays. Thus, after exposure for 3 days to a mixture of 1 part of lecithin emulsion to 600 parts of culture fluid, a dose of X rays three and a half times the normal was needed to kill the animals. It is, of course, of interest that the substances concerned are considered to be important constituents of the cell membrane. However, it must be kept in mind that animals kept in emulsions of this sort for some days may change their nutritive condition. Giese and Heath (1948) have shown the importance of the nutritive condition for sensitivity to X rays. Likewise, cholesterol and lecithin may have a protective action of the sort found by Evans *et al.* (1942) for sea-urchin sperm.

Halberstaedter and Back (1943) found that pretreatment of *Paramecium caudatum* with sublethal concentrations of colchicine for 2 days lowered the resistance to X rays. In controls, the dose required to produce immediate death of 50 per cent of the animals lay between 250,000 and 300,000 r, while in the colchicine-treated animals it was between 100,000 and 150,000 r. They found no effect of colchicine on resistance to arsenic or ultraviolet. No explanation is offered for these findings.

*Effect on the Medium.* There is considerable evidence that radiation may act indirectly on cells by way of an effect upon the medium surrounding them. To what extent, then, can these effects on the medium account for the total effect of radiation upon the cell? It seems obvious that this is not a matter of mutually exclusive alternatives. Rather, it is a question of the relative importance, under the conditions employed, of different mechanisms by which the radiation effect could be brought about. All discussion of effects on the medium will be included in this section even though other than lethal effects are involved.

Taylor *et al.* (1933) found that an irradiated tap-water extract of commercial yeast killed *Colpidium campylum* when added after irradiation. They were able to demonstrate the presence of hydrogen peroxide in the irradiated water in concentrations sufficient to kill, and concluded that this substance probably played a major role in the death of irradiated protozoa, although they added that production of other toxic agents by irradiation of the yeast medium was not improbable. Since that time, several workers have reported that their media were not rendered toxic to unirradiated paramecia by doses of X rays sufficient to kill directly irradiated organisms (Piffault, 1939; Back and Halberstaedter, 1945; Giese and Heath, 1948; Wichterman, 1948a). However, Piffault (1939) reported that medium exposed to a dose about four times as great as that necessary to produce death of irradiated animals was toxic and gave positive tests for peroxide. Giese and Heath (1948) reported that medium irradiated with 1,000,000 r was not toxic, while only 560,000 r, directly to the animals, led to complete death within 75 min. It would seem then that the lethal effect of the radiation cannot be ascribed to stable poisonous substances produced in the medium. Obviously, an unstable poison of short half life is not excluded, since an appreciable time had to elapse between the end of irradiation and the addition of cells to the medium.

Mention may also be made here of a report by Heilbrunn and Young (1935) which states that eggs of sea urchins irradiated in the presence of minced ovarian tissue are more affected by X rays (cleavage delay) than eggs irradiated free of such materials. They believe that the irradiated ovarian material produced poisonous substances. This is in line with Loofbourow's finding (1948) of similar injurious substances from yeast and other organisms. Heilbrunn and Young were unable to demonstrate the production of such substances by organs other than ovaries.

The problem of lethal substances in the medium has been carefully investigated with sea-urchin sperm by two groups of workers (Evans *et al.*, 1942; Evans, 1947; Barron *et al.*, 1949a, b). Evans *et al.* (1942) showed that the effect of X rays on *Arbacia* sperm as measured by the percentage of fertilized eggs was markedly influenced by dilution of the sperm and by the addition of various protective substances to the medium in which irradiation was carried out. The more dilute the sperm suspension during irradiation, the more effective was a given dose of X rays. A wide variety of substances, such as egg albumin, gelatin, and egg water protected against X rays if present during the irradiation. No effect of protective substances was found on cleavage delay by irradiated sperm. Tests for hydrogen peroxide suggested that too little was formed to account for the effects. The investigators accepted Fricke's activated water as an explanation of the effects.

On the other hand, Evans (1947) comes to the conclusion that hydrogen



peroxide can account for at least a part of the effects at very high doses. He shows that both the percentage of eggs fertilized and the time of first cleavage were affected by treating sperm with hydrogen peroxide in about the concentration in which it is found in heavily irradiated sea water. Contrary to the conclusion of Evans *et al.* (1942), cleavage delay is therefore not necessarily a direct effect. Evans (1947) believed that the effect of hydrogen peroxide was slow, so that by irradiating a dilute suspension of sperm and removing the sperm quickly to fresh medium all or almost all this effect could be eliminated. The peroxide effect appears to be different from the "activated water" effect in a number of respects such as the effect on cleavage as well as on fertilizing power of the sperm. Consequently, both these mechanisms of indirect action through the medium have to be taken into account.

Barron *et al.*, (1949a, b) have also studied this problem with *Arbacia* sperm by using respiration of the sperm to measure the radiation effect. Diluted sperm (1:200) showed that X rays, even at doses as low as 100 r, caused a measurable inhibition of respiration. These investigators pointed out that hydrogen peroxide in low concentration increased respiration and so the effect at low doses could not be caused by this substance. Furthermore, they found that sea water exposed to 100,000 or 200,000 r had a marked inhibitory effect on sperm respiration, and that the addition of catalase to the water before addition of sperm had no effect. Finally, they were not able to demonstrate any hydrogen peroxide in sea water exposed to 200,000 r although such water inhibited respiration by about 60 per cent. They believe that stable organic peroxides which may be formed in sea water can account for this and other cases in which irradiated fluids have an effect.

Attention should be drawn here to the finding of Stone and his coworkers (Wyss *et al.*, 1950) that mutagenic substances are formed by ultraviolet irradiation of culture medium.

In summary, there is a good deal of evidence that stable substances which produce biological effects can be formed in the medium as a result of irradiation. However, these substances do not appear to be formed in sufficient concentration to account to any large extent for such effects as death of paramecia, since medium treated with a dose which would have been lethal to the animals is not in itself lethal. Evans *et al.* (1942) have presented evidence for the formation of very unstable substances in the medium; and such substances may be responsible for at least part of the effects produced. This group was unable to find evidence that cleavage delay was affected by such unstable substances. It can be concluded that stable and unstable substances produced in the medium all play a role but that direct effects in the cells are probably also involved. The relative importance of these diverse pathways of action of the radiation may not be the same for different effects.

## RETARDATION OF CELL DIVISION

Giese (1947a) has reviewed in detail much of the work on the effects of radiation on cell division, and Hevesy (1945) has presented a review from a rather different point of view. Nevertheless, it seems desirable to summarize the major work on fission delay in the protozoa and cleavage delay in invertebrate eggs and to expand, somewhat, particular topics upon which the reviewer wishes to express opinions. It has been fully established by many investigators that ultraviolet and ionizing radiations, in sufficient dosage, retard cell division. In some cases at least, this retardation may last for several divisions; but, unless death intervenes, recovery of the normal rate occurs sooner or later. Perhaps this recovery is one of the most interesting features of the effect.

There are a few cases in which visible light has been reported to retard division. Most of these are the result of photodynamic action (Blum, 1941, may be consulted for a review of this phenomenon). Tennent (1942) mentions delay in cleavage in the sea urchin by visible light in the presence of several photodynamic dyes. Giese (1946a) reports delay in cell division in *Paramecium caudatum* in the presence of eosin and in the ciliate *Blepharisma*, which contains a naturally occurring photodynamic pigment. However, Zhalkovsky (1938) claims a reduction of cell division in *Paramecium caudatum* by visible light in the absence of a photodynamic dye. The delay was said to be more marked in direct than in reflected light. Phelps (1946) reports that the division rate of cultures of the colorless *Tetrahymena geleii* was lowered by exposure to sunlight. This has since been shown to result from destruction of necessary substances in the medium (Phelps, 1949). Perhaps a somewhat similar interpretation would be possible for Zhalkovsky's results.

There have been a number of purported cases of acceleration of division by small doses of radiation. Giese (1947a) reviews these cases and comes to the conclusion that most of the evidence is of questionable significance. However, he apparently accepts several reports, mainly from the older literature, of acceleration by ionizing radiation. In all cases, the effects are small, and careful statistical analysis has not been made. Moreover, there would seem to be considerable inherent difficulty in being sure that there are no systematic differences between the controls and the experimentals other than in the exposure to radiation. Further investigation seems necessary before accepting stimulation of division by low doses of radiation as a real phenomenon.

Although division delay is an extremely common result of irradiation, it is not universal. Halberstaedter and Luntz (1929) and Halberstaedter and Back (1942) were unable to find division delay in *Eudorina* or *Pandorina* at any sublethal dose of radium rays or X rays.

*Recovery.* As far as the reviewer is aware, there is no adequate evidence



that division delay by radiation ever lasts for more than a few divisions, provided the cells survive at all. The cases in microorganisms in which lasting reduction in rate of multiplication have been found are probably the results of genetic changes quite independent of the original retardation of division.

The time course of recovery may vary greatly in different cases. In some cases, retardation may last for several divisions before complete recovery occurs; in some, recovery may be complete, or nearly so, by the first division, while in others, there seem to be stages during which no recovery occurs. The studies have been concerned mainly with qualitative and quantitative descriptions of the time course of recovery with only a small amount of attention being devoted to attempts to influence recovery experimentally.

The most complete quantitative study of division delay has been carried out with echinoderm eggs and sperm, chiefly those of *Arbacia*. Most of the investigations with ionizing radiations have been concerned with the first cleavage only. However, Miwa *et al.* (1939a) irradiated unfertilized eggs of *Pseudocentrotus depressus* with  $\beta$  particles from radon and recorded the time to both the first and the second cleavage. The data suggest to the reviewer that the interval between the first and second cleavages may be slightly longer than normal at higher doses although the authors say that "there is little or no delay in the . . . second division" (see Fig. 8-1a). Yamashita *et al.* (1939) exposed fertilized eggs of this same sea urchin to X,  $\gamma$ , and  $\beta$  rays and stated that they could find no evidence that irradiation during most of the period before the first division had any marked effect on later cleavages. However, Blum . . . Loos (1949) mention in an abstract that they have obtained the same results with X rays and with ultraviolet radiation in fertilized *Arbacia* eggs. Presumably, this includes delay in cleavages later than the first. The method used by Blum and his coworkers (see Blum and Price, 1950) is probably better designed to detect small differences in cleavage times than were those of previous investigators. It therefore seems probable that the effects of ionizing radiations last for more than one cleavage but are, in most experiments, of small importance in intervals beyond the first interval following the treatment.

The recovery of sea urchin eggs from cleavage delay by ionizing radiations has been investigated by two groups of workers, a Japanese group (Miwa, Mori, and Yamashita) and Henshaw and his collaborators. Henshaw's results have been interpreted theoretically by Lea (1938a, b, 1947).

Henshaw (1932, 1940c) and Miwa *et al.* (1939a) found that the longer the period between irradiation of eggs and insemination with unirradiated sperm, the less the effect. In other words, recovery occurred between irradiation and insemination. Irradiation of sperm also brings about

cleavage delay, but there is no recovery if sperm are kept for a time before they are used for insemination (Henshaw, 1940a; Miwa *et al.*, 1939a; Mori *et al.*, 1939). The cleavage delay produced by irradiated sperm can be shown to increase as a linear function of the dose. According to Lea (1947), the data of Henshaw (1940a) show an increase of 25 min in cleavage delay for each doubling of the dose.

Lea (1938a, b) shows that the recovery in eggs can be adequately represented by an exponential decay of the original effect with time according to the expression  $e^{-t/T}$ , where  $t$  is the time after irradiation and  $T$  is a constant. The value of  $T$  was calculated to be 35 min for the fertilized egg and 104 min for the unfertilized egg.

For *Arbacia*, Henshaw (1940b) has shown that there is little if any delay in stages before the prophase of the first cleavage. Most of the delay is in the prophase, with minor delays in the later stages of mitosis. Yamashita *et al.* (1940) find major delays in the late nuclear fusion and prophase stages for *Pseudocentrotus* and *Strongylocentrotus*. Henshaw and Cohen (1940) irradiated eggs at different times after fertilization and found that the effect produced by a given dose increased for the first 10–15 min and then declined, so that by the end of prophase there was little if any effect on the time of the first cleavage. The decline was not quite regular, there being a small secondary increase in sensitivity at about 25 min (early prophase). Henshaw and Cohen (1940) show that there is good agreement between the first peak in sensitivity and changes in viscosity and permeability, but point out that recovery in the egg pronucleus up to the time of fusion might also be involved. Lea (1947) also suggests that the early increase in sensitivity is due to a recovery process which he believes can continue up to prophase. Thus the time for recovery decreases as the time after insemination increases. The later drop in sensitivity may be due to irreversible changes leading to division which cannot be affected by radiation; but, as pointed out by Henshaw and Cohen (1940), this explanation fails to account for the secondary peak.

Henshaw (1940d) has shown that low temperature (0°C) decreases the rate of recovery. Mori *et al.* (1939) found no effect of dilution of sperm immediately after irradiation, and conclude that failure of the sperm to recover is not due to something produced in the medium by the radiation.

Recovery of the sort reported for *Arbacia* apparently does not occur for all invertebrate eggs. Henshaw *et al.* (1933) treated *Cumingia* and *Arbacia* eggs simultaneously with X rays. The *Arbacia* eggs showed recovery but the *Cumingia* eggs did not. It should be noted that recovery, in the sense that the egg developed successfully, did occur. The *Cumingia* eggs simply showed the same delay in first cleavage whether the dose of X rays was given at low intensity over a long time or at high intensity for a brief period. Cook (1939) has also reported no recovery for *Ascaris equorum* eggs exposed to X rays and then kept at 5°C for



periods of time ranging up to six months. The eggs kept in the cold

showed the same delay in the first few divisions as those allowed to develop immediately at 25°C. However, another effect, production of abnormal embryos, showed recovery during the period in the cold. Evans (1950) has confirmed these results but has found a somewhat different situation with *Arbacia* eggs. *Arbacia* eggs irradiated with low-intensity X rays divide without further delay when the irradiation ceases, which suggests that recovery and inhibition occur at nearly equal rates. However, the effects on later embryonic development are more pronounced after longer exposures, indicating that the rates of recovery for the two effects are quite different and that recovery of division delay is more rapid.

There have been a number of reports that marine eggs exposed to ultraviolet show delay in cleavages later than the first one after treatment. Chase (1937, 1938), using a quartz mercury arc and the eggs of the marine worm *Urechis caupo* and the sand dollar *Dendraster excentricus*, found that several successive divisions were affected when radiation was given before fertilization. Not all of Chase's data demonstrate recovery but observations were extended over only the first few cleavages. Giese (1938c), using monochromatic ultraviolet, found the same thing for *Strongylocentrotus purpuratus*. Sample cases from Chase and Giese are shown in Fig. 8-1a. However, Giese (1939b) irradiated the sperm of

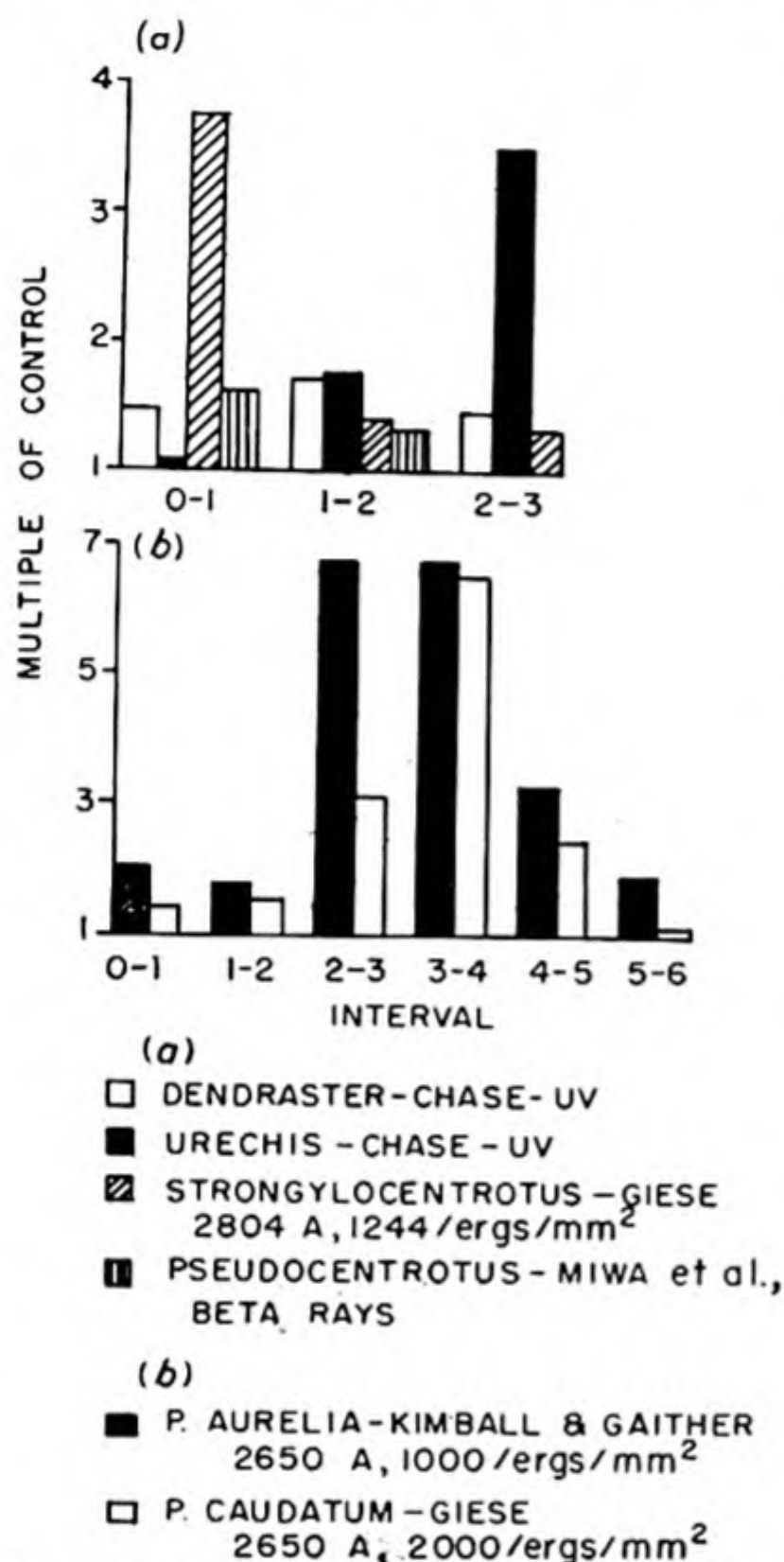


FIG. 8-1. Bar diagrams to show the relative importance of delay in various division intervals after irradiation. The data from the various authors was recalculated as time for the division interval in question and this time was expressed as a multiple of the control time for the same interval. (a) Data (Chase, 1938; Miwa et al., 1939a; Giese, 1938c) for the first three cleavages of various marine eggs. The eggs were irradiated shortly before insemination, and 0-1 is the interval between insemination and the first cleavage. (b) Data for two different species of *Parametium*. The interval 0-1 is between irradiation and the first division thereafter. (Giese, 1945b; Kimball and Gaither, unpublished.)

*S. purpuratus* and obtained retardation of first cleavage with, at most, a very slight effect on later divisions. Marshak (1949b) reports that sperm exposed to 2537 Å ultraviolet delay the first cleavage but have no appreciable effect upon the second. He also reports that there is less delay in division if the sperm are irradiated shortly before insemination than if they are irradiated  $\frac{1}{2}$ –1½ hr before. Perhaps this increase of the effect with time between irradiation and insemination was due to some substance produced in the medium.

Blum and Price (1950) report a detailed study of recovery in *Arbacia* eggs irradiated with ultraviolet from a mercury arc. In most cases, the

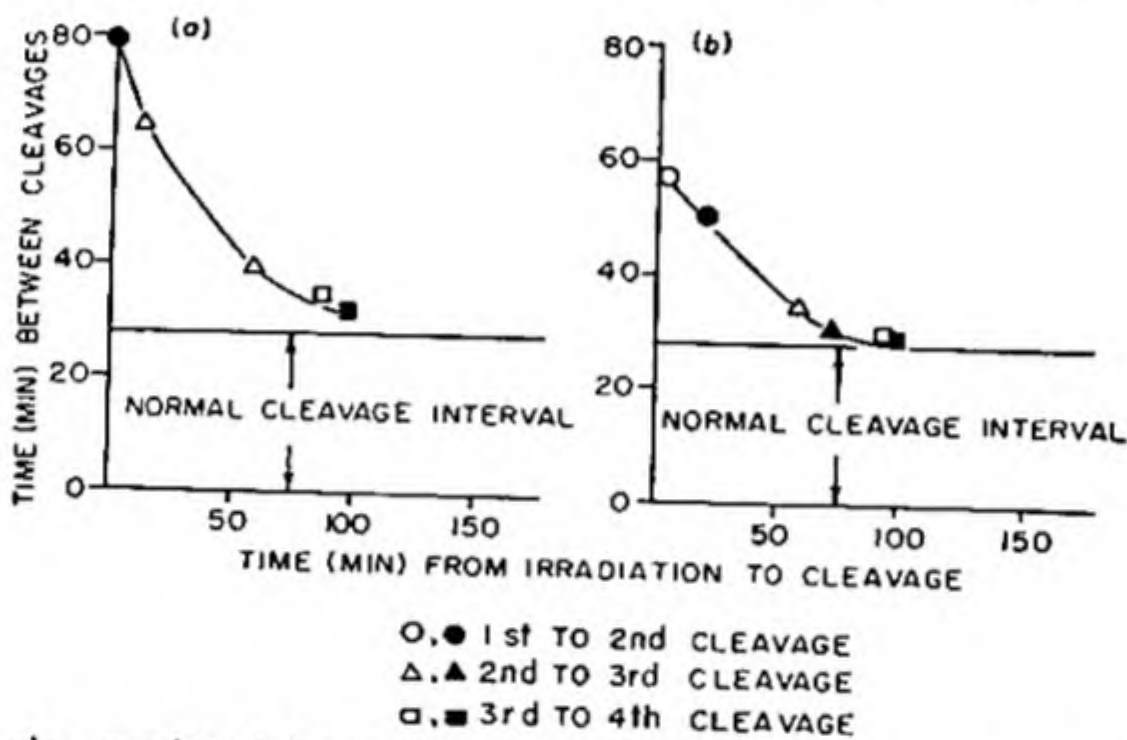


FIG. 8-2. Graphs, modified from Blum and Price (1950), to show the recovery of *Arbacia* eggs from the effects of ultraviolet irradiation. Eggs were irradiated at various times and the time in minutes between a given pair of cleavages was plotted against the time from irradiation to the cleavage beginning the interval in question. The open and solid symbols represent different experiments in which radiation was given at different times. The points are approximately the center of distribution of a whole series of points given by Blum and Price (1950). (a) Irradiation after the first cleavage. (b) Irradiation before the first cleavage.

eggs were irradiated after fertilization, often after the first cleavage. Eggs irradiated early in a cleavage interval showed a maximum effect on the duration of that interval, while those irradiated late in an interval showed no effect until the succeeding intervals. By plotting the length of the interval against time from irradiation to the cleavage beginning the interval, a smooth curve showing recovery from the effect was obtained (see Fig. 8-2). The smooth form of this curve and its extension over more than one division interval suggests that the recovery process was independent of the occurrence of cleavage. Recovery was also demonstrated to occur in eggs irradiated before cleavage. Blum . . . Loos (1949) state in an abstract that X-irradiated eggs behave in the same way as those exposed to ultraviolet, but they give no details.

The results of Blum and Price (1950) are in agreement with those of



Henshaw and Cohen (1940) in that sensitivity decreases as the time after fertilization increases. However, Henshaw and Cohen found a small secondary increase in sensitivity in the early prophase stage. Gross (1950) found that *Chaetopterus* eggs exposed to ultraviolet showed somewhat the same sensitivity relations as did X-irradiated *Arbacia* eggs. The eggs were quite sensitive for the first 30 min, after which the sensitivity decreased but increased again in the period from 40 to 50 min. The simplest explanation of the results of Blum and Price (1950) would be that there is a period after which division is irrevocably determined. However, the results of the other two investigators are not so easily explained, and it seems probable that rather complex factors are involved in these changes in sensitivity.

These reports make it clear that spontaneous recovery from the effects of radiation occurs in the sea urchin. For ultraviolet, it appears established that this recovery is a gradual process extending over more than one division. Both Henshaw and Lea treat the X-ray data as though recovery were complete by the time division occurs, but Henshaw and his coworkers do not present evidence on later cleavages. In view of this and the statement of Blum . . . Loos (1949), that X- and ultraviolet-irradiated material showed the same behavior, it seems possible that in both cases the recovery process is independent of the occurrence of cleavage. If this is so, as suggested also in a brief statement in Blum *et al.* (1951), then radiation must affect something which controls the rate at which division occurs instead of simply destroying some material which must be restored to its original amount before any cleavage can take place.

The separation between cell division and recovery is much more marked in the ciliate protozoa. Giese (1939a, 1945b) has shown clearly for *Paramecium caudatum* that ultraviolet retards several divisions following the irradiation (see Fig. 8-1b). Giese and Reed (1940) have shown the same thing in somewhat less detail for several species of *Paramecium*. Giese (1946a) has made similar findings for the retardation of division by visible light in *Paramecium* exposed to eosin, and in *Blepharisma*. Kimball *et al.* (1952) find the following pattern for *P. aurelia* exposed to monochromatic ultraviolet (see Fig. 8-1b). The same pattern has been found for wave lengths 2378, 2537, 2650, and 2804 Å. The first division following irradiation is usually markedly delayed. The next division is also delayed, but less so. Either the third or fourth interval is often extremely long, lasting in some cases for two weeks or more. Finally, recovery of the normal rate is usually complete by the sixth division. It seems possible that at least three processes should be recognized: (1) retardation of the first division after irradiation, (2) a long but not permanent cessation of division, usually setting in after two or three divisions have occurred, and (3) a relatively small increase in all other division intervals through about the sixth. The relative magnitude of these various effects appears

to change with dose, the second process becoming relatively more important as the dose increases. However, even at quite low doses, effects lasting through about six divisions can be recognized. Recovery from lower and higher doses appears to require about the same number of divisions.

The effect of X rays on cell division in the ciliates has been investigated to only a small extent, mainly because the doses necessary to produce an appreciable delay are of the order of a hundred thousand roentgen units. Perhaps it is this feature more than any other which emphasizes the great radioresistance of protozoa, for most other cells are retarded by much smaller doses. Back (1939) reports that the X-ray dose for death within 2 hr in *P. caudatum* lies between 400,000 and 600,000 r. A dose between two-thirds and five-sixths the lethal dose leads to a permanent cessation of division. About half the lethal dose leads to a retardation of the first division of some 36 to 48 hr after which the normal rate is restored, apparently with no effect on divisions later than the first. Giese and Heath (1948) report an effect only on the first division at lower doses but effects on later divisions at higher doses. Powers and Shefner (1950) report that 650,000 r reduces by half the rate at which irradiated *P. aurelia* reaches the first division, but effects on later divisions are not mentioned. Kimball *et al.* (1952) found an effect of X rays on divisions later than the first in *P. aurelia*, but the effect was much smaller, relative to delay in the first division, than that for ultraviolet.

Thus the results for *Paramecium* and the sea urchin seem quite different. A guess might be made that cleavage delay in the sea urchin corresponds more nearly to the delay in the first division in *Paramecium*, while the other delays in this organism have no counterpart in the sea urchin. However, it is not quite certain that the latter is true. In *Paramecium*, the four products of the first two divisions of one treated animal may have cessation periods of rather different duration (Kimball *et al.*, 1952). In the sea urchin, a similar occurrence would lead to abnormal cleavage, and perhaps development would finally stop. A number of workers have reported abnormal cleavages following irradiation of invertebrate eggs (see Giese, 1949, for review).

Mention may be made here of the investigations of Robertson (1935a, b) with the flagellate *Bodo caudatus*. She found that continuous exposure to  $\gamma$  rays from radium led, at first, to a decrease in rate of cell division, but later a partial recovery toward the normal rate occurred even though the irradiation continued. Meanwhile, the flagellates became larger than normal in size. Following cessation of irradiation, they multiplied more rapidly for a time before the normal rate was restored. The partial resistance to radiation did not persist.

*Sensitivity.* There has been a rather miscellaneous group of investigations on the effects of various factors on retardation of cell division by



radiation. Alpatov and Nastiukova (1934c) found that the effect of ultraviolet on *Paramecium* was intensified by exposure to slightly unfavorable temperatures after irradiation, the least effect being evident when the animals were irradiated at temperatures near the middle of the vital range. They suggest that these changes in sensitivity may be related to protoplasmic viscosity. In another paper (1934b) they report that sodium sulfate and electrical stimulation, both of which increased the viscosity, decreased the effect of ultraviolet while potassium thiocyanate and mild narcosis, which decreased the viscosity, increased the sensitivity to ultraviolet. On the other hand, Wilbur and Recknegel (1943) have shown that treatment of *Arbacia* eggs with potassium citrate (0.35 M) only slightly decreased the retardation of cleavage by X rays, whereas addition of calcium or magnesium to the sea water had no effect at all. All these treatments affected the viscosity of the egg. These investigators also report that doses of X rays (30,000 r) which markedly affected the rate of cleavage had no detectable effect on viscosity. They concluded that changes in ionic composition and viscosity cannot be important factors in division delay by X rays. Zirkle (1936) has shown that a high carbon dioxide content in the atmosphere at the time of irradiation increases the sensitivity of *Paramecium* to the division-retarding effects of X rays. Hutchings (1948) reported that *Arbacia* eggs suffer cleavage delay when briefly exposed to 36°C 10 min after insemination. She found that the cleavage delays produced by this temperature and by 2537 Å ultraviolet were additive, or nearly so, when the eggs were exposed to the high temperature either before or after the irradiation.

The phenomenon of photoreactivation has been studied for cleavage delay in sea urchin eggs exposed to ultraviolet by Blum . . . Robinson (1949); Blum, Loos, and Robinson (1950); Blum, Robinson, and Loos (1950); Marshak (1949a, b); and Wells and Giese (1950). It has also been found for division delay in ultraviolet-irradiated *P. aurelia* by Kimball and Gaither (1951). The subject will not be discussed further here, since it will be reviewed in detail by Dulbecco in Chap. 12 of this volume.

Differences in sensitivity between various strains and species have been reported by several investigators. Alpatov and Nastiukova (1934a) found distinct differences in sensitivity to ultraviolet between *P. caudatum* and *P. bursaria*. Giese and Reed (1940) made an extensive study of different species and stocks of *Paramecium* and found considerable difference in their sensitivity to the division delay produced by ultraviolet. They also found that starved paramecia were more susceptible than well-fed ones. Giese (1946b) reported a wide range in sensitivity to ultraviolet in the eggs and sperm of different marine invertebrates. The echinoderms, whose eggs have indeterminate cleavage, are mature when shed, and cleave radially, showed a considerable difference in sensitivity between egg and sperm, and cleaved abnormally only when given high

doses. The other organisms were from various phyla whose eggs have determinate cleavage, are immature when shed, and cleave spirally. They showed little difference in sensitivity between the egg and sperm and showed irregular cleavage at low doses. Henshaw *et al.* (1933) report marked differences in sensitivity to cleavage delay by X rays between *Chaetopterus*, *Nereis*, *Cumingia*, and *Arbacia* eggs.

*Localization.* A considerable amount of evidence in regard to the part of the cell which is responsible for radiation-induced cleavage delay has been accumulated. It rather strongly suggests a nuclear effect for the sea urchin but is not so clear for the protozoa. Of course, the delay need not be due to the same causes in such different types of cells, although such a unifying hypothesis would be attractive. Thus the details of the mitotic process in the ciliate protozoa are quite different from those in the sea urchin. Moreover, in the eggs, there is no growth in size between cell divisions, whereas in ciliates growth occurs, and is, perhaps, essential for later divisions.

In the sea urchin, it has been repeatedly shown that irradiation of either the sperm or the unfertilized egg can bring about cleavage delay. Among reports on this subject may be mentioned those of Henshaw and Francis (1936) and Henshaw (1940a, b) on X-irradiated *Arbacia*; Marshak (1949b) and Blum, Robinson, and Loos (1950) on *Arbacia* exposed to ultraviolet; Giese (1939b, c; 1946b) on a variety of marine invertebrates exposed to ultraviolet. The two gametes obviously contribute quite differently to the zygote. Thus the sperm contributes the male pronucleus and the centrosome which functions in the first cleavage (Henshaw and Francis, 1936). The egg contributes the female pronucleus and the bulk of the cytoplasm. As Henshaw and Francis (1936) point out, the delay produced by irradiation of the unfertilized egg indicates that injury to the centrosome is not involved since this gamete does not contribute a functional centrosome. The one portion of the zygote to which both gametes are known to contribute is the nucleus. Therefore, the simplest conclusion would be that the effect is on this structure. This conclusion is not absolutely demonstrated by such evidence since it is possible that the sperm contributes cytoplasmic elements which, though small in bulk, are important in division. Nonetheless, the very fact that irradiation of either gamete produces delay certainly suggests a nuclear effect. This conclusion is not affected by the difference in sensitivity between egg and sperm, for the nuclei in the two gametes are in quite different physical states and are subject to different amounts of shielding in the case of ultraviolet.

Supporting evidence for a nuclear site of the injury is furnished by experiments with eggs fragmented by centrifugation. Henshaw (1938) has shown that X irradiation of either the whole *Arbacia* egg or the nucleated half results in cleavage delay but X irradiation has no effect on



enucleate halves subsequently fertilized with unirradiated sperm. Blum, Robinson, and Loos (1950, 1951) carried out similar experiments with ultraviolet-irradiated *Arbacia* eggs and found the same results. They also demonstrated that other combinations, such as irradiated sperm with unirradiated enucleate halves of eggs, result in delay. Their conclusion was that the locus of the primary injury must be in the nucleus. Harding and Thomas (1949, 1950) found that centrifuged *Arbacia* eggs irradiated unilaterally with ultraviolet through the fat cap were more affected than were those irradiated through the pigmented end. They draw no final conclusions from these results, but a nuclear effect seems to be favored since the nucleus would be displaced toward the fat cap. Marshak (1949b) suggests that the relative inefficiency of ultraviolet for the egg as compared to the sperm favors a nuclear effect. Otherwise, the high proportion of ultraviolet absorbed in the cytoplasm should make the egg more, not less, sensitive.

Thus most of the evidence clearly favors a nuclear effect. However, there is certain evidence which is not in full agreement. Giese (1939b, 1947a) has shown that the action spectrum for delay by ultraviolet-irradiated sperm resembles the absorption spectrum for nucleoprotein, whereas that for ultraviolet-irradiated eggs resembles the absorption spectrum for certain other proteins (Fig. 8-3). Giese (1939b, 1947a) discusses various explanations among which is the possibility that the effect is partially cytoplasmic in the case of the egg but entirely nuclear for the sperm. However, in the egg, the primary absorption might be in the cytoplasm with secondary effects on the nucleus or it might be by proteins, other than nucleoproteins, in the nucleus. Since the nuclei in the two gametes are not in the same state, such differences in importance between nucleic acid and protein absorption would be possible. This emphasizes that action spectra cannot be used to reach a clear decision between a nuclear and a cytoplasmic site of radiation injury since nucleic acids and several kinds of proteins are present in both.

Blum and Price (1950) believe that the fact that recovery from ultraviolet-induced delay is independent of the occurrence of cleavage suggests a cytoplasmic locus, since the nucleus undergoes major changes at the time of cleavage. However, Blum, Robinson, and Loos (1950, 1951) present evidence that the primary absorption of the ultraviolet is in the nucleus. On the basis of their belief that the sperm cannot be photoreactivated before fertilization, they conclude that recovery is a cytoplasmic process. The argument that cytoplasmic rather than nuclear processes are suggested by a recovery independent of cleavage appears weak since the cytoplasm at cleavage may well undergo changes quite as profound as those in the nucleus. The evidence that sperm are not subject to photoreactivation has been called in question by the finding of Wells and Giese (1950) of some photoreactivation of *Strongylocentrotus* sperm. Blum,

Robinson, and Loos (1951) do not believe that this applies to *Arbacia*. Thus there are compelling reasons for thinking that cleavage delay is due to nuclear damage. The evidence that recovery depends on cytoplasmic events is suggestive but not very strong.

The situation for the protozoa is not so clear. Holweck and Lacassagne (1931a, b) found that one of the effects which occurred in the flagellate *Polytoma uvella* when it was exposed to  $\alpha$  particles was a cessation of division accompanied by an increase in cell size. The cells failed to recover from this effect and finally died, so it is not clear that the effect should be classified with division delay. Holweck and Lacassagne do not give detailed data but state that the effect was due to a single-particle event

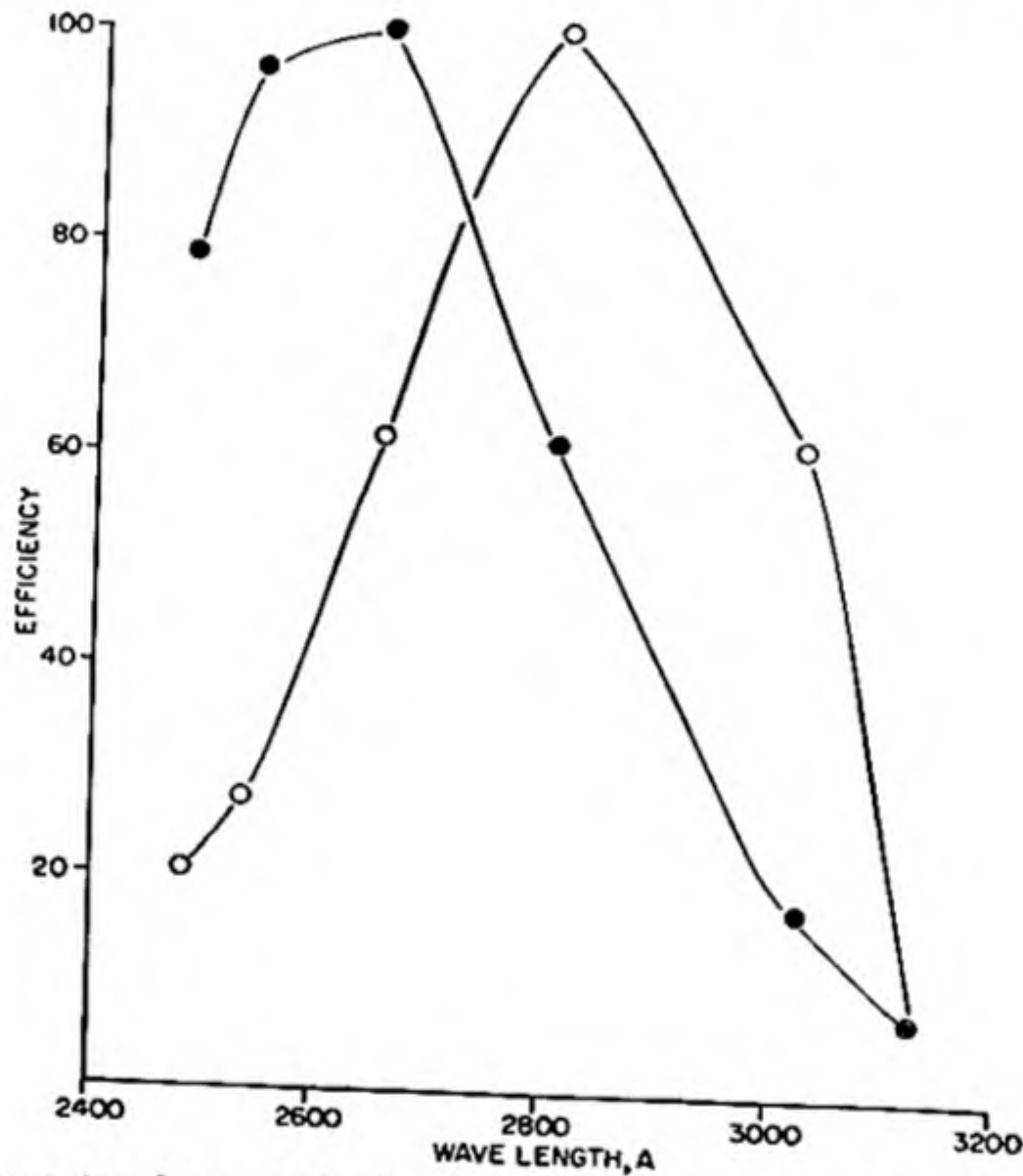


FIG. 8-3. Action spectra for retardation of cleavage in the sea urchin for irradiated sperm and irradiated eggs, replotted from Giese (1947a). Solid circle = sperm irradiated. Open circle = egg irradiated.

and that sensitive volume calculations suggested a body the size of the centrosome. Certainly, it would be expected that injury to the centrosome might lead to difficulties with division; but in the absence of detailed data it is hard to judge how compelling is the evidence for this identification.

Using *Amoeba proteus*, Mazia and Hirshfield (1951) have found evidence for both nuclear and cytoplasmic effects on division delay by ultraviolet. The nucleated halves of bisected amoebae are more sensitive to division



delay by the radiation than are whole amoebae. This cannot be interpreted as the result of shielding of the nucleus by the larger amount of cytoplasm in whole amoebae, for both whole and half amoebae spread over the substrate so that they are of approximately the same thickness. Mazia and Hirshfield (1951) suggest that the increased sensitivity reflects an effect of the cytoplasm on recovery processes. They also find evidence for a cytoplasmic effect of the radiation in the fact that irradiated enucleate halves die more rapidly than the unirradiated halves.

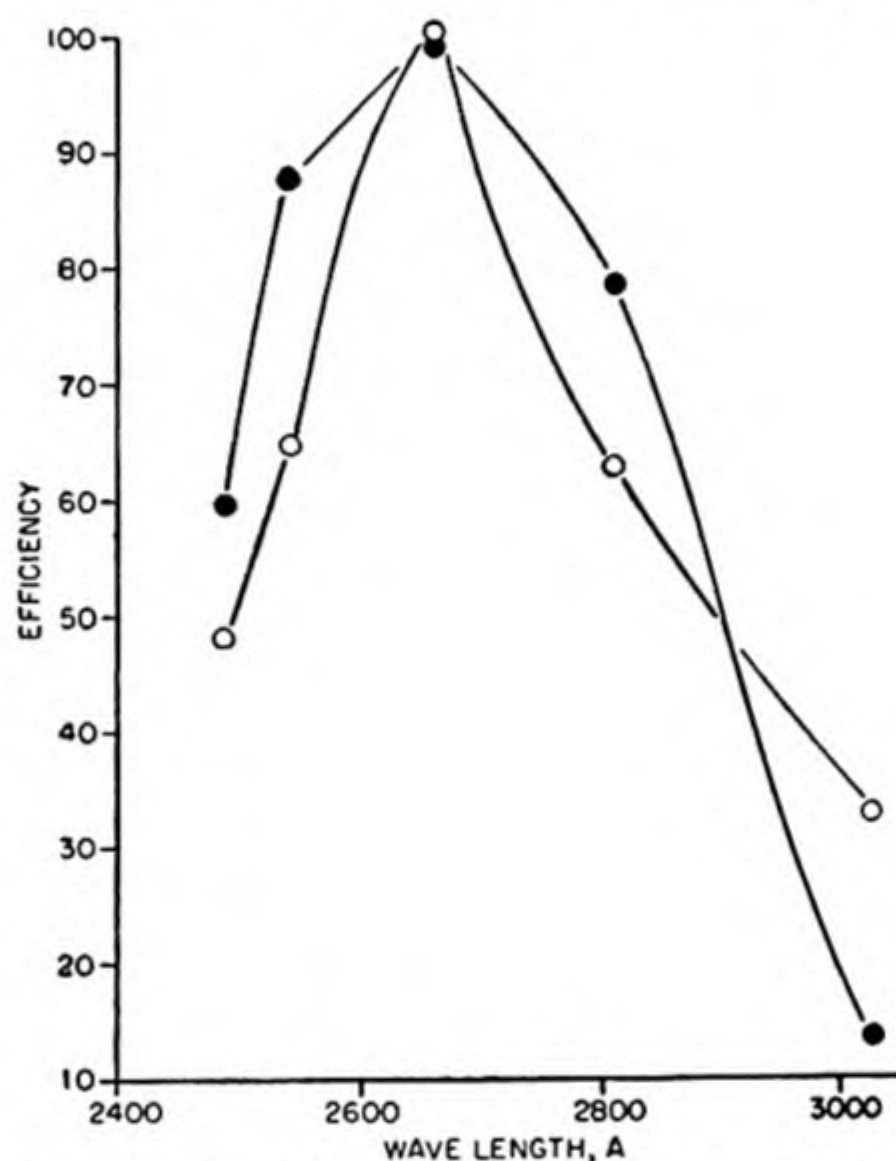


FIG. 8-4. Action spectra for retardation of cell division in *Paramecium*, modified from Giese (1945b). Solid circle = time to third division—starved. Open circle = time to recovery—well-fed.

For *Paramecium*, the evidence as to localization of the effect is not adequate. Giese (1945b) has found an ultraviolet action spectrum for retardation of the third division in starved *P. caudatum* similar to the absorption spectrum for nucleoproteins (Fig. 8-4). A similar action spectrum was found for recovery of the normal rate in well-fed animals. However, the action spectrum for time to the third division in well-fed animals is rather nondescript, having a very slight maximum at 2804 Å (Fig. 8-5). Kimball *et al.* (1952) have been able to confirm, although with differences in detail, the nondescript action spectrum for well-fed *P. aurelia* (Fig. 8-5) but have been unable to demonstrate a nucleoprotein-like spectrum for recovery of the normal rate. Giese (1947a) concludes that "the immediate effect is upon the cytoplasm but the more lasting effect is upon the nucleus." It does not seem to the reviewer that this

conclusion is justified. As has been pointed out in a preceding paragraph, a nucleoprotein-type action spectrum does not, by itself, indicate that the nucleus is involved. Effects upon cytoplasmic nucleic acids are just as possible. Moreover, the duration of an effect through a number of divisions before recovery does not necessarily mean that the effect is nuclear. Long-lasting cytoplasmic effects are also possible. It can only be concluded that there is no critical evidence on the localization of the changes leading to division delay in *Paramecium*.

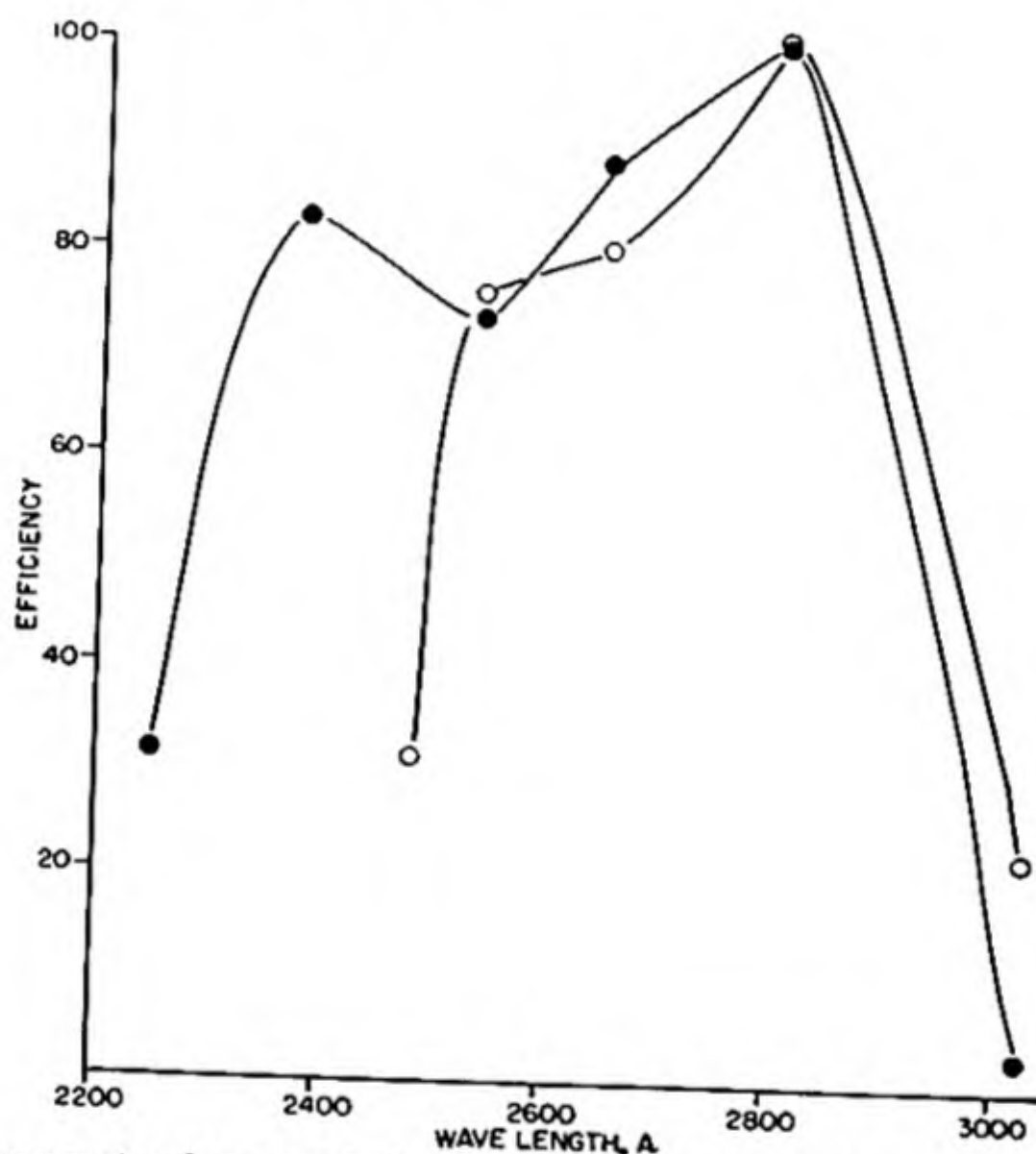


FIG. 8-5. Action spectra for retardation of cell division in well-fed paramecia, modified from Giese (1945b), and from Kimball, Geckler, and Gaither (1952). Open circle = time to third division (Giese)—*Paramecium*, well-fed. Solid circle = time to sixth division (Kimball, Geckler, and Gaither)—*Paramecium*, well-fed.

### INHERITED EFFECTS

There have been scattered reports of mutation induction in invertebrates, other than insects, but such studies add nothing basically new to the studies of mutation which are to be considered elsewhere in this series. This section will be concerned almost entirely with the protozoa. For this group a rather different point of view from that in classical mutation investigations must be taken where inherited effects are concerned. Work reviewed by Sonneborn (1947, 1949) has made it clear that watch must be kept for kinds of inherited differences which are not dependent on



differences in the genes for their maintenance. In the protozoa, inheritance in lineages of cells multiplying vegetatively as well as inheritance over the sexual processes can be investigated. Thus, in addition to the classical mutation approach, other lines of investigation may prove fruitful. Actually, the investigations available for this review have added only scattered bits of information along these lines.

To the reviewer's knowledge, MacDougall (1929, 1931) was the first to report the induction by radiation of inherited changes in protozoa. She exposed mass cultures of the ciliate *Chilodonella uncinatus* to ultraviolet from a quartz mercury arc for brief periods on several successive days. In some cultures abnormal animals appeared. A few of the abnormalities proved to be inherited for many generations of asexual reproduction, and in some cases through conjugation. These included apparent tetraploid and triploid forms as well as others exhibiting only changes in form and size. Since the mutant forms appeared in only a few of the irradiated cultures, the conclusion that they were induced by the radiation can hardly be considered established, especially since the number of control cultures is not given, but the aim of this work was to obtain mutant forms, not to investigate their origin.

Mottram (1941, 1942) exposed cultures of an amiconucleate *Colpidium* (said in a footnote to belong to this genus though called *Paramecium* in most of the text) to daily doses of ultraviolet or  $\gamma$  radiation (also to low and high temperatures and to carcinogenic hydrocarbons). After 4 to 62 days, a few abnormal animals were found. The doses of ultraviolet are not given. The doses of  $\gamma$  rays ranged from 800 to 12,160 r. The abnormalities continued to produce abnormal descendants though not necessarily of the same type. Somewhat similar changes were produced by ultraviolet irradiation of *Glaucoma setosa*. Investigations with *Paramecium caudatum* and *Aspidisca* sp. are also reported, but it is not stated that radiation was employed.

Mottram (1942) suggests that changes in viscosity are involved, that the changes are cytoplasmic, not nuclear, and that they are similar to those involved in carcinogenesis in higher forms. The arguments Mottram gives for cytoplasmic and not nuclear change are inconclusive. Nonetheless, it is hard to see how chromosomal changes could be involved in an amiconucleate *Colpidium*. However, further investigation is required of both the origin and inheritance of these changes. It is especially necessary that the experiments permit a quantitative study of the origin of the abnormalities followed by a careful genetic analysis of the inheritance.

Spencer and Calnan (1945), working with *P. multimicronucleatum*, report a long-term deleterious effect of continuous sublethal exposures to radium, to a number of dyes, and to methylcholanthrene. The animals were grown in mass culture with continuous exposure to the agent.

Transfers were made every 10–12 days. The division rate was about one per day. Although the exposed cultures continued to multiply through many transfers, they eventually died out while control cultures survived. Thus the cultures exposed to radium succumbed at the 190th transfer while 16 control series were still alive after more than 206 transfers. It would appear that the effects of sublethal exposure to radium and the other agents were accumulative over the course of many generations of cell division. However, no genetic analysis of the material was made, and the method allows autogamy, and so gene recombination, to occur. Various complex processes of selection of both the paramecia and their accompanying bacteria are also possible. An interpretation in terms of mechanism seems impossible without further analysis.

Schaeffer (1946) reported an inherited change in size induced in the giant multinuclear ameba (*Chaos chaos*). Some of the amebae broke into fragments following X irradiation. The largest fragments developed into clones of normal size and the smallest died. However, some of the medium-sized fragments grew into clones whose average volume was about 60 per cent of the parent clone. One such clone was maintained for four years. When this small clone was exposed to X rays, clones were obtained which were still smaller. These latter clones had been maintained for three months at the time of the report. Schaeffer does not propose a mechanism to explain these results. In the light of the multinuclear condition of this species, it seems difficult to suppose that gene mutations or chromosomal aberrations were involved. If fragmentation into medium-sized pieces is really a necessary first step, interesting speculations concerning the determination of size in such multinucleate protoplasmic masses might be made. However, no data on the frequency with which the change has occurred are given, so that it is difficult to evaluate the apparent correlation between the size of the fragment and the occurrence of the variant.

The self-reproducing cytoplasmic particle, kappa, of *P. aurelia* has been shown to be inactivated by X rays (Preer, 1948, 1950), by nitrogen mustard (Geckler, 1949), and by 2537 Å ultraviolet (Kimball, 1950). Preer (1950) finds that the curve of the logarithm of the number of particles against dose is not quite linear and suggests several sources of difficulty which might explain the departure, since he believes that the inactivation of kappa is basically a single-event phenomenon. Depending on the interpretation, the true inactivation dose (37 per cent dose) is considered to lie between 3400 and 4000 r or at approximately 10,000 r. Sensitive volumes calculated on this basis are in reasonable agreement with the size of the particles which can be observed under the microscope. Preer reports that microscopic examination showed that it takes some 2 to 3 days in the absence of cell division for the visible kappa particles to disappear following X irradiation.



Dippell (1948) has reported finding spontaneous mutations of kappa. So far no reports of radiation-induced mutations of this entity have been made.

Lee (1949) found that X irradiation of *P. bursaria* led to changes in mating type which became more frequent as the dose was increased. He suggests that X rays may induce autogamy which may in turn cause an increased frequency of mating type change. However, as he points out, the connection between change of mating type and autogamy has not been fully established for this species. Thus some other pathway of action of the X rays is possible.

A series of reports on genetic changes in *P. aurelia* induced by  $\beta$ , ultra-violet, and X radiation and by nitrogen mustard have been made by Geckler, Kimball, and Powers and their coworkers. The method used by all these workers was basically the same and depended on the fact that autogamy makes the animals completely homozygous. The paramecia were exposed to the radiation, and a number of the exposed animals were isolated. After a period of vegetative multiplication autogamy was induced, and a number of autogamous animals were isolated from the progeny of each treated animal. Each autogamous animal was allowed to multiply for a period of several days and was then checked for survival (Powers) or for survival and amount of growth (Kimball, Geckler). The percentage surviving with normal growth can be taken as a measure of the effect.

When ionizing radiations were used, it was found that at doses much too low to have immediately detectable effects either on survival or rate of division of vegetative animals, many of the autogamous clones were not viable or, if viable, divided more slowly than usual. The dose range used has been from about 300 to about 20,000 r; above the latter dose almost all the exautogamous clones were affected. Such observations have been reported by Kimball (1949a, b) for  $\beta$  particles from  $P^{32}$  outside the culture medium and for X rays, by Powers (1948) for  $P^{32}$  and for a mixture of  $Sr^{89}$ ,  $Sr^{90}$ , and  $Y^{90}$  in the medium, and by Powers and Shefner (1948, 1950) for X rays. Geckler (1950) reported similar findings for nitrogen mustard; Powers and Shefner (1950) and Powers and Raper (1950) reported on doses of X rays and nitrogen mustard which were sufficiently high to have a distinct immediate effect on the animals in addition to the effect after autogamy. Kimball and Gaither (1951) report that doses of 2650 A ultraviolet, which are sufficient to produce a detectable effect after autogamy, also cause a temporary retardation of the first few cell divisions following irradiation.

The simplest interpretation for effects which do not appear until after autogamy is that they are due to gene mutations or chromosomal aberrations in the micronuclei. Sonneborn has shown that autogamy results in the formation of a completely homozygous synkaryon from which the new

macronuclei and the micronuclei of the exautogamous clone are derived. Sonneborn also has given evidence that the macronucleus is a compound structure in which each gene and chromosome are represented many times. The evidence is reviewed by Sonneborn (1947). Under the circumstances, mutations or chromosomal aberrations would not be expected to express themselves immediately following irradiation but only after homozygosity of both the macro- and micronuclei had been brought about by autogamy.

On this basis and from the results of several breeding experiments, Kimball (1949b) came to the conclusion that most of the death and low rate of multiplication in the exautogamous progeny of irradiated animals were the results of gene mutations or chromosomal aberrations. Kimball (1949a) showed that a given total dose of  $\beta$  particles divided into several small daily fractions, with the animals undergoing several cell divisions between each fraction, was as effective as the same dose given in a single exposure of a half-hour's duration or less. This was taken to mean that the mutational changes must have been gene mutations or one-break chromosomal aberrations rather than two-break aberrations. However, the dosage curve was more nearly typical of a "multiple hit" than a "one-hit" curve. For this reason, Kimball (1949a) suggested that most of the non-normal exautogamous clones were the result of the combined action of a number of mutant genes with individual effects too small to be detected.

Powers and Shefner (1948) using X rays and Geckler (1950) using nitrogen mustard have both presented further evidence from breeding experiments for the genic or at least the micronuclear basis of the post-autogamous effect. However, Geckler (1950) reported on a number of findings which can be explained in terms of micronuclear inheritance only with great difficulty if at all. Kimball (1949b) reported one case of inheritance which did not conform to expectations.

Another phenomenon, not at present explained, is that reported by Powers and Shefner (1950) for very high doses of X rays and by Powers and Raper (1950) for nitrogen mustard. In both cases, they found that death after autogamy rose to a maximum as the dose increased, then declined somewhat. With X rays, there was a secondary rise at very high doses.

The reviewer believes that there is strong evidence that radiations and nitrogen mustard induce mutations in the micronuclei of *P. aurelia* and that these mutations express themselves in death and low rate of multiplication of exautogamous clones. However, there is rather convincing evidence that this is not the whole story and that other phenomena may also play a significant role. Further experiments to define this situation more thoroughly are needed.

Most of the work with *Paramecium* has concerned itself more with the



nature of the inherited changes which are produced than with the mechanism by which they are produced. The work of Powers (1948) with radioactive isotopes in the medium is an exception. He found that for equal activities, as measured by an air ionization chamber and vibrating reed electrometer,  $P^{32}$  was four to six times as effective in producing death after autogamy as a mixture of  $Sr^{89}$ ,  $Sr^{90}$ , and  $Y^{90}$ . It would be expected that the phosphorus would be concentrated in the nucleus but not the strontium and yttrium. Rubin (1948) computed from Powers' data the expected increase of specific ionization due to the concentration of phosphorus in the nucleus and came to the conclusion that it could not account for the total difference in effect which Powers found. He concluded that some other factor must be involved and was inclined to believe that it was the transmutation phenomenon, i.e., the result of the radioactive disintegration of phosphorus atoms incorporated in the molecules of the chromosomes. A number of approximations of necessity enter the calculations so that it would seem well to withhold final judgement until further investigations of this matter have been made.

In summary, nuclear mutations have been induced in *Paramecium aurelia* and are subject to quantitative study. In addition, several ill-defined inheritable changes have been found after irradiation of various protozoa. Further advances in understanding these changes must depend on obtaining them in a situation in which definitive genetic analysis is possible.

### MISCELLANEOUS EFFECTS

*Activation of Eggs.* Loeb (1914) discovered that unfertilized eggs of *Arbacia* and *Chaetopterus* could be stimulated to begin parthenogenetic development by exposure to ultraviolet from a quartz mercury vapor lamp. Giese (1949) has recently reviewed the subject but a brief discussion of it seems desirable here.

The activation of the sea urchin egg has been studied by several investigators. This egg is mature when shed and activation is indicated by membrane elevation and cleavage. The later cleavages in activated eggs may be quite irregular, with spindle abnormalities and fragments of chromatic material on the spindle (Nebel *et al.*, 1937). Hollaender (1938) has shown that wave lengths of 2650 Å and longer have very little effect on the whole *Arbacia* egg. The curve of effectiveness rises sharply around 2400 Å and is still rising at the shortest wave length used, 2260 Å (Fig. 8-6). This type of curve is a rather generalized one, resembling the absorption curves for certain proteins and for lipids. Giese (1949) believes that it may be the result of absorption in the lipids of the cell membrane. It would appear that the curve for different eggs may not be the same. Giese (1938d) found no activation by 2537 Å ultraviolet, in the doses used, of the eggs of the sea urchin *Strongylocentrotus*, but he (1939c)

readily activated eggs of the marine worm *Urechis caupo* with this same radiation.

Harvey and Hollaender (1938) fractionated the *Arbacia* egg by centrifugation into white (nucleated) and red (nonnucleated) halves and separated the latter into yolk and pigment quarters. Activation was obtained with some differences in detail when either half or either of the two quarters were exposed to ultraviolet of 2480 Å or below. Activation was also obtained for the red half and its two quarters with doses of the band of wave lengths 2650–3000 Å, which were ineffective with the whole egg or the white half. It can be concluded that the nucleus does not play an important role in activation by ultraviolet. While there seem to be differences in detail in the activation of different parts of the egg, it is not at all unlikely that changes in the surface of the eggs are involved. This is further indicated by the studies of Reed (1943) and Spikes (1944) which have shown localized effects on membrane formation as the result of unilateral irradiation of sea urchin eggs. Similarly, Tchakotine (1935a, b) has shown localized changes in the surface followed by activation phenomena as a result of localized irradiation of the *Pholas* egg.

The investigations of Heilbrunn and Wilbur (1937) and Wilbur (1939) on the effects of calcium and magnesium on ultraviolet activation of the *Nereis* egg also suggest a surface phenomenon. Heilbrunn and Wilbur (1937) have shown that citrate inhibits the activation; they suggest that this is due to removal of calcium from the cortex, so preventing the calcium release which, on Heilbrunn's views, is responsible for the activation. Alsup (1941) found that photodynamic activation of *Nereis* eggs by visible light in the presence of eosin or rose bengal was likewise inhibited by citrate. Wilbur (1939) has shown that magnesium inhibits activation by small doses of ultraviolet but the inhibition is overcome by larger doses. Calcium antagonized this action of magnesium. It was suggested that magnesium acts in the same manner as calcium but less efficiently.

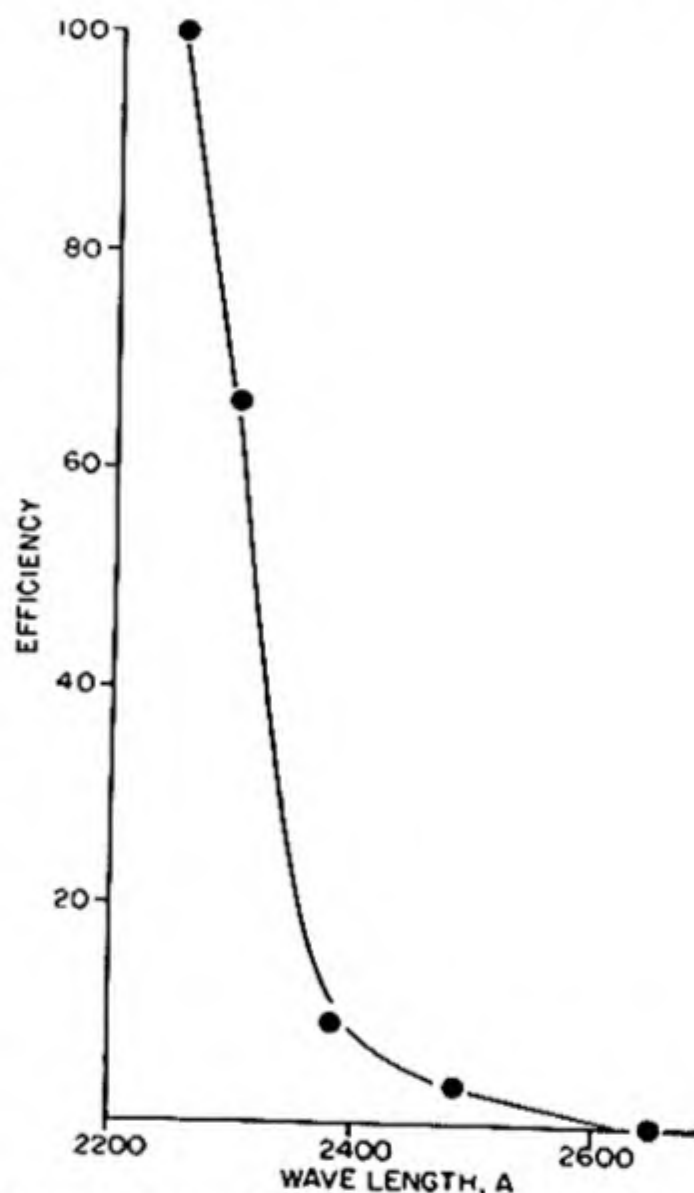


FIG. 8-6. Action spectrum for activation of *Arbacia* eggs, from Hollaender (1938).

*Excystment of Protozoa.* The process of excystment in the ciliate pro-



tozoan *Colpoda steinii* is under excellent experimental control. Exposure to a special medium results in complete excystment, starting at about 2 hr after exposure and ending in less than 1 hr from its inception. Taylor *et al.* (1936) have shown that a dose of 38,400 r of X rays given at the rate of 1280 r/sec within the first 60 min after exposure of the ciliates to the excystment medium increases the time to 50 per cent excystment to about 420 min. The same dose given 120 min after exposure to the excystment medium has very little effect. From the form of the curves of percentage excysted against time, it is concluded that, in the period between 60 and 120 min, there is a mixture of sensitive and resistant cysts and some in a transitional state between sensitive and resistant. The cysts in this transitional state are apparently more easily prevented from excysting by the X rays than are cysts in the other two states.

Giese (1938a, 1941, 1945a), using the same techniques, has shown that monochromatic ultraviolet also increases the time to excystment. The action spectrum has been determined for wave lengths from 2537 to 3660 Å. There is a small peak at 2804 Å suggestive of the absorption spectrum of certain proteins. The dose of 2654 Å ultraviolet to double excystment time is approximately 600 ergs/mm<sup>2</sup>.

*Motility and Behavior of Protozoa.* There have been a number of observations on the behavior and motility of protozoa during or immediately following irradiation. Giese and Leighton (1935a; Giese, 1938b, 1945a) have presented a series of quantitative observations on the effect of ultraviolet on a variety of ciliates. In particular, Giese (1938b) gives comparative data for 50 per cent rotation on the long axis and 50 per cent immobilization for *Tetrahymena glaucomiformis*, *Colpidium colpoda*, *Stylonychia curvata*, *Paramecium bursaria*, *P. aurelia*, *P. caudatum*, and *P. multimicronucleatum*, *Blepharisma undulans*, *Spirostomum ambiguum*, *Bursaria truncatella*, and *Fabrea salina*. Giese (1945a), using monochromatic ultraviolet, has shown that the action spectrum for immobilization and for ciliary reversal in *Paramecium* has a peak at 2804 Å, suggesting that it is similar to the absorption spectra for certain proteins (Fig. 8-7). A variety of other studies on motility and behavior are summarized in Table 8-2.

Immobilization has been observed often and in many cases is probably a sign of impending death. However, this is not necessarily so. The reviewer (unpublished) has observed complete immobilization of *P. aurelia* by 2250 Å ultraviolet at a dose (1000 ergs/mm<sup>2</sup> incident on the quartz container) which not only is not lethal but has only a very small effect on the time to the first division after irradiation.

Wichterman (1948a, b) has reported in some detail the effect of X rays on the mating reaction in *P. bursaria* and *P. calkinsi*. Doses in the range between 100,000 and 700,000 r lessen or prevent the mating reaction and the pair formation which usually follows from it. Apparently, pair for-

mation is somewhat more easily affected than is the mating reaction itself. At nonlethal doses, recovery from these effects seems to be possible. *Paramecium calkinsi* is affected by somewhat smaller doses than *P. bursaria*.

**Sensitization to Heat.** Bovie and Klein (1919) first reported that paramecia could be made more sensitive to heat by exposure to ultraviolet. Giese and his coworkers have made a detailed investigation of this phenomenon. Giese and Crossman (1945a) may be consulted for reports of work with other organisms.

The time to death at a single lethal temperature,  $42^{\circ}\text{C}$ , was used as a measure of the resistance to heat. The possibility that the minimum lethal temperature was changed was not tested. Giese and Crossman

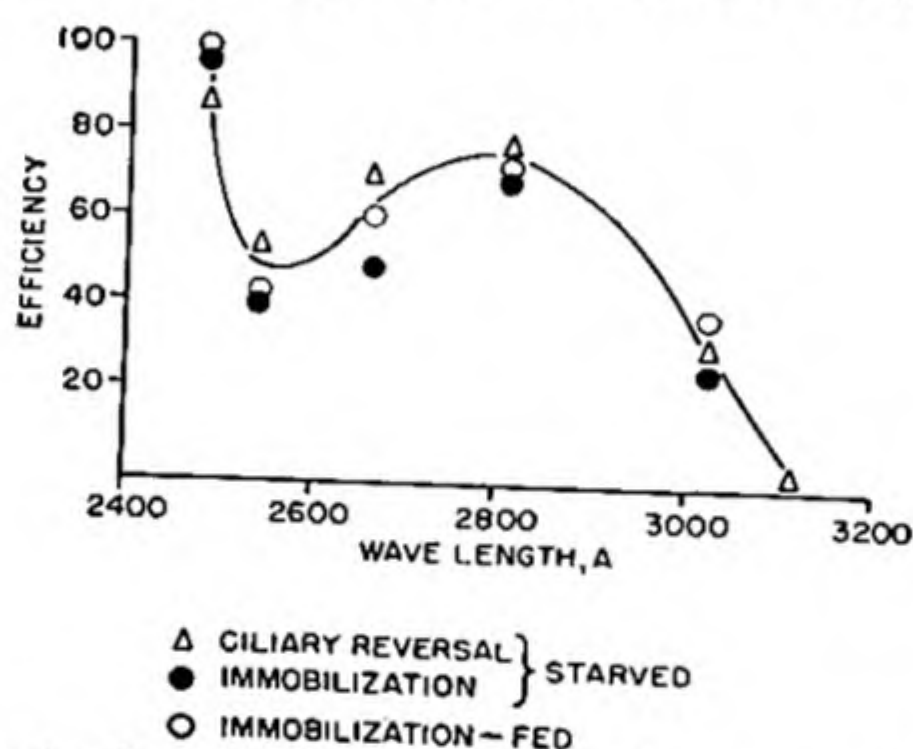


FIG. 8-7. Action spectra for immobilization and ciliary reversal in *Paramecium*, from Giese (1945a).

(1945a) investigated the action spectrum for heat sensitization of *Paramecium*. They found that 2483 Å, the shortest wave length used, was much more effective than 2537 Å or still longer wave lengths. There was a small secondary peak at 2804 Å. The dose to reduce the time to death at  $42^{\circ}\text{C}$  to half its control value can be calculated to be about 400 ergs/mm<sup>2</sup> for 2483 Å and about 1800 ergs/mm<sup>2</sup> for 2804 Å.

They also found that recovery from the effect occurred. For all wave lengths other than 2654 Å the course of recovery was much the same, and it was complete in about 4 days. Animals exposed to 2654 Å recovered much more slowly and recovery was only about two-thirds complete by 4 days. Giese and Crossman suggest that this means that more than one material is involved and that the slow recovery from 2654 Å is due to a larger component of effect on nucleoproteins than at the other wave lengths.

Giese and Crossman (1945b) have shown that visible light in conjunction with a photodynamic dye can increase the sensitivity of *Paramecium*



to heat. Giese (1946a) has shown that visible light by itself can do so for the ciliate *Blepharisma*, which contains a natural photodynamic pigment.

Giese and Heath (1948) demonstrated that paramecia can be sensitized to heat by sublethal doses of X rays. They emphasize that dosage calculations with the thin window tube employed are subject to much question but give 56,000 r/min as an approximate rate. On this basis, it can be estimated from their data that doses between 100,000 and 300,000 r are necessary, depending on the nutritive state of the animals, to reduce the time for death at 42°C to half. Well-fed, rapidly dividing animals are more sensitive than starved ones. This finding contrasts with Giese and Reed's finding (1940) that well-fed paramecia are more resistant to the division-retarding effects of ultraviolet than are starved ones. Thus one cannot speak of a general resistance to radiation but only resistance to specific effects of specific radiations. Recovery from heat sensitization was shown to occur when the paramecia were fed after irradiation but not when they were starved. Giese and Heath (1948) conclude that recovery must involve the synthesis of new materials by the cell.

Giese and Crossman (1945b) suggest that radiation partially denatures proteins. Exposure to heat is then supposed to complete the denaturation and so lead to death more rapidly than in animals not exposed to radiation. Giese (1947b) found evidence for sensitization of nucleoproteins to heat by ultraviolet. Partially purified nucleoprotein from *Strongylocentrotus* sperm, dissolved in 2 M sodium chloride, forms threads when poured into dilute sodium chloride. Brief exposures to ultraviolet (mainly 2537 Å) followed by a 10-min exposure to 80°C changed the nucleoprotein so that the threads did form. The reverse procedure, exposure to 80°C and then to ultraviolet, had no effect.

*Miscellaneous Microscopically Visible Changes.* Microscopically visible changes in the nucleus of the cell are treated by Carlson (Chap. 11, volume I of this series). Some of the more recent observations of various changes in both the nucleus and cytoplasm in protozoa and invertebrate eggs are summarized in Table 8-3. No attempt has been made to include papers in which microscopic observations were merely incidental to other work. As has been pointed out in a previous section, many of these changes may be those that occur in dying cells.

*Various Physiological, Biophysical, and Biochemical Effects.* For the most part, the work on protozoa and invertebrate gametes has not been directed to a study of the enzyme systems affected by the radiation, the colloidal changes in protoplasm, the changes in permeability of membranes, and alterations in the chemical composition of the cell. Information of an indirect sort on some of these matters has been mentioned in preceding sections of this chapter but direct investigations are scarce and are listed briefly in Table 8-4 without discussion in the text.

TABLE 8-1. KILLING OF PARASITIC PROTOZOA BY RADIATION

Organism	Radiation	Criterion	Dose	Reference
<i>Trypanosoma brucei</i>	Soft X rays	Decreased infective power	6,000 r	Patel (1936)
	Ultraviolet	Decreased infective power	$\frac{1}{8}$ – $\frac{1}{16}$ H.S.E.	Patel (1936)
<i>Trypanosoma gambiense</i>	X rays	Visible change	100,000 r	Halberstadter (1938)
<i>Trypanosoma cruzi</i>	X rays	Failure to infect	12,000 r	Emmett (1950)
		No visible change	100,000 r	
		Decreased infective power	10,000 r	
<i>Plasmodium gallinaceum</i> sporozoites	Ultraviolet*	Failure to infect	Not given	Russell <i>et al.</i> (1941)
<i>Plasmodium gallinaceum</i> endothelial stages	X rays	No effect on infection	150–700 r	Zain (1943)
<i>Plasmodium gallinaceum</i> sporozoites	X rays	Failure to infect	8,000 r	Bennison and Coatney (1945)
<i>Plasmodium gallinaceum</i> trophozoites	X rays	Failure to infect	20,000 r	
		Rate of development of the infection	4,000 to 8,000 r	
<i>Plasmodium malaria</i> trophozoites	X rays	Failure to infect	5,000 r	Bennison and Coatney (1945)
<i>Plasmodium lophurae</i> trophozoites	X rays	Failure to infect	16,000 r	Rigdon and Rudisell (1945)
<i>Eimeria tenella</i> oocysts	Ultraviolet	No infection of chicks	2 units	Fish (1932)
		100 per cent mortality <i>in vitro</i>	1 unit	
<i>Eimeria tenella</i> oocysts	X rays	Failure to infect	13,500 r	Albanese and Smetana (1937)
		Effect on severity of infection	9,000 r	
<i>Eimeria tenella</i>	X rays	Distinct effect on severity of infection	9,000 r	Waxler (1941)
<i>Eimeria perforans</i>	Ultraviolet	Some infection	13,500 r	Litwer (1935a, b)
<i>Eimeria stiedae</i>		Inhibition of <i>in vitro</i> development of oocysts	Not given	
<i>Adelina deronis</i>	X rays	Abnormal oocysts	1,000 r	Hauschka (1944)
<i>Endamoeba histolytica</i> cysts	Ultraviolet	Decrease in number excysting	Not given	Stoll <i>et al.</i> (1945)
<i>Endamoeba histolytica</i> trophozoites	X rays	Partial inhibition of growth <i>in vitro</i>	60,000 and 120,000 r	Sadun <i>et al.</i> (1950)
		Infectivity unchanged	120,000 r	

\* Unless otherwise stated in this and other tables "Ultraviolet" means the radiation from a quartz mercury arc presumably with major output at 2537 Å.



TABLE 8-2. MOTILITY AND BEHAVIOR OF PROTOZOA

Radiation	Organism	Effect	Dose	Reference
X rays.....	<i>Paramecium</i>	Acceleration of movement followed by slowing, spinning, and immobilization	$<5 \times 10^5$ r	Dognon and Piffault (1931a)
X rays.....	<i>Paramecium caudatum</i>	Irregular swimming, immobilization	1 to $6 \times 10^5$ r	Back and Halberstaedter (1945)
X rays.....	<i>Paramecium bursaria</i>	Acceleration Retardation Immobilization	$1 \times 10^5$ r $2 \times 10^5$ r $1 \times 10^6$ r	Wichterman (1948a)
X rays.....	<i>Paramecium calkinsi</i>	Retardation	$4 \times 10^5$ r	Wichterman (1948b)
X rays.....	<i>Trypanosoma gambiense</i>	A few nonmotile	$2 \times 10^5$ r	Halberstaedter (1938)
X rays.....	<i>Pandorina</i>	Immobilization	3 to $6 \times 10^5$ r	Halberstaedter and Back (1942)
Neutrons...	<i>Euglena</i>	Clear zone at top of culture (change in motility?)	1250 n	Jennings and Garner (1947)
$\gamma$ (radium)...	<i>Physarium polycephalum</i>	Plasmodium moves more slowly Plasmodium moves away from needle	Not given	Seifriz (1936)
$\alpha$ (polonium)	<i>Polytoma uwelli</i>	Immobilization	Not given	Holweck and Lacassagne (1931a, b)
2804 A ultraviolet	<i>Paramecium multimicro-nucleatum</i>	50 per cent immobilization	$1.1 \times 10^4$ ergs/mm <sup>2</sup>	Giese and Leighton (1935a)
2537 A ultraviolet	<i>Paramecium multimicro-nucleatum</i>	50 per cent rotation	$1.8 \times 10^4$ ergs/mm <sup>2</sup>	Giese and Leighton (1935a)
Intense flashes of ultraviolet	<i>Paramecium multimicro-nucleatum</i>	Avoiding reaction, retardation, rotation, immobilization	Not given	Rentschler and Giese (1941)
Ultraviolet...	<i>Paramecium caudatum</i>	Acceleration of contractile vacuoles, retardation of food vacuole formation	Not given	Roskin and Shishliaeva (1933)
Ultraviolet...	<i>Spirostomum ambiguum</i>	Various effects	Not given	Shirley and Finley (1949)
Ultraviolet...	<i>Amoeba proteus</i>	Various effects of several salts	Not given	Black (1936)
Visible plus photodynamic dye	<i>Trypanosoma gambiense</i> and <i>T. evansi</i>	Immobilization	Not given	Levaditi and Prudhomme (1945)

TABLE 8-3. MICROSCOPICALLY VISIBLE CHANGES INDUCED BY RADIATION

Radiation	Organism	Effect	Reference
$\gamma$ (radium).....	<i>Physarium</i>	Protoplasmic streaming, viscosity, distribution of granules	Seifriz (1936)
$\gamma$ (radium).....	Opalinid ciliates	Distribution of mitochondria and vegetative granules	Horning (1937)
X rays.....	<i>Unio tumidus</i> eggs	Changes in appearance of nuclei due to coagulation and permeability changes	Wottge (1939)
Ultraviolet.....	<i>Unio tumidus</i> eggs	Coagulation within nucleus	Wels (1938)
Ultraviolet.....	<i>Amoeba proteus</i>	Form and general appearance	Black (1936)
2537, 2654, 2804, 3025 A 2650, 2804 A	<i>Paramecium multimicronucleatum</i> <i>Paramecium aurelia</i>	Vesiculation	Giese and Leighton (1935a) Kimball (1949c)
Ultraviolet.....	<i>Kahlia simplex</i>	Formation of large, vacuolated bodies in the macronucleus	Kimball and Gaither (1951)
Ultraviolet.....	<i>Spirostomum ambiguum</i>	Double animal formation	Horvath (1947)
Ultraviolet.....	<i>Spirostomum ambiguum</i>	Fragmentation of macronucleus, various cytoplasmic changes	Shirley and Finley (1949)
Ultraviolet, microbeam	<i>Amoeba proteus</i> , <i>Amoeba verrucosa</i> , <i>Paramecium caudatum</i>	Localized coagulation in cytoplasm	Tchakotine (1935c)
Ultraviolet, microbeam	<i>Paramecium caudatum</i> , <i>Spirostomum ambiguum</i> , <i>Amphileptus claparedei</i>	Localized colloidal changes in macronucleus	Tchakotine (1936)
Ultraviolet, intense flashes	<i>Paramecium multimicronucleatum</i>	Change in shape, vesiculation	Rentschler and Giese (1941)
Ultraviolet, intense flashes	<i>Amoeba proteus</i> , <i>Amoeba dubia</i> , <i>Euglena</i> , <i>Volvox</i> , <i>Chilomonas</i> , <i>Stylonychia</i> , <i>Chilodonella</i> , <i>Coleps</i> , <i>Urocentrum</i> , <i>Paramecium bursaria</i> , <i>Epistylis</i> , <i>Stentor</i> , <i>Bursaria</i> , <i>Frontonia</i>	Various changes, mainly fragmentation	Harvey (1942)
Visible light plus photodynamic dye	<i>Amoeba dubia</i>	Blister formation, hyaline areas formed, pseudomembranes formed	Hyman and Howland (1940)
Visible light plus photodynamic dye	<i>Lytechinus</i> eggs	Variety of nuclear and cytoplasmic changes	Tennent (1942)



TABLE 8-4. BIOCHEMICAL AND BIOPHYSICAL EFFECTS

Effect	Organism	Radiation	Reference
Increased permeability at low doses Decreased permeability at high doses	<i>Barnea candida</i> egg	$\beta$ and $\gamma$ (radium)	Simon (1939)
Increased permeability of nuclear membrane	<i>Unio tumidus</i> egg	X rays	Wottge (1939)
Change in permeability . . . .	<i>Paramecium</i>	Ultraviolet	Roskin and Shishliaeva (1933)
No detectable change in permeability at doses which retard cleavage	<i>Strongylocentrotus</i> egg	Ultraviolet 2483, 2537, 2654, 2804, 3130 Å	Reed (1948)
No detectable change in permeability at doses which retard cleavage	<i>Arbacia</i> egg	X rays 10,000 r	Lucké <i>et al.</i> (1951)
Changes in viscosity . . . . .	<i>Amoeba proteus</i> and <i>A. dubia</i>	Photodynamic action	Alsup (1942)
No effect on viscosity at doses which markedly retard cleavage	<i>Arbacia</i> eggs	X rays	Wilbur and Recknegel (1943)
Changes in viscosity . . . . .	<i>Spirostomum</i>	Ultraviolet	Shirley and Finley (1949)
No effect on respiration at doses causing early death	<i>Arbacia</i> and <i>Chaetopterus</i> eggs	X rays	Chesley (1934)
No effect on respiration . . . .	<i>Barnea candida</i> egg	$\beta$ and $\gamma$ (radium)	Simon (1939)
50 per cent inhibition of respiration	<i>Arbacia</i> sperm	X rays 10,000 to 20,000 r	Barron <i>et al.</i> (1949a, b)
Decreased assimilation of lipid and glycogen	<i>Paramecium</i>	Ultraviolet	Roskin and Shishliaeva (1933)
Changes in the amount of ammonia produced per cell	<i>Bodo audatus</i>	$\gamma$ (radium)	Lawrie and Robertson (1935)
Free fat formed in the cytoplasm	<i>Amoeba proteus</i> and <i>A. dubia</i>	Ultraviolet	Heilbrunn and Daugherty (1938)
Destruction of red pigment	<i>Blepharisma</i>	Visible light	Giese (1938e); Giese and Zeuthen (1949)
Change in the mineral constituents of protoplasm . .	<i>Paramecium</i>	X rays	Berner (1942)
Destruction of the enzymes for oxidation of acetate and succinate	<i>Arbacia</i> sperm	X rays	Barron <i>et al.</i> (1949a, b)
More rapid killing by sulfhydryl inhibitors	<i>Paramecium</i>	Visible light	Calcutt (1950)

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## CHAPTER 9

# Radiation and Viruses\*

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*Introduction. Effect of radiations on virus infectivity: Ionizing radiations—Ultra-violet radiation—Visible light. Differential effect of radiations on various properties of viruses: Nonlethal effects—Separation of properties of inactive virus particles. Irradiated virus in multiple and mixed infection: Interference phenomena—Reactivation phenomena. Intracellular irradiation of viruses: Irradiation of cells infected with exogenous viruses—Radiation and latent viruses. References.*

### 1. INTRODUCTION

To few fields of biology have radiation studies contributed as much as they have to virology. The reasons are to be found in the properties of viruses and in the methodology of virus research. A virus can be defined as a submicroscopic entity capable of self-reproduction after exogenous infection of specific living cells (Luria, 1950). According to host, viruses may be classified as animal viruses, plant viruses, and bacterial viruses or bacteriophages. In their extracellular state, virus particles have characteristic sizes and chemical compositions, a common feature of which is the presence of protein and of nucleic acid of either the ribose or the deoxy-ribose type. Their small size, nucleoprotein content, and ability to reproduce inside cells make them useful models for the study of reproduction of genetically specific biological units. At the same time they offer unique opportunities for observation both in the resting, extracellular state, in which they can be submitted to a variety of treatments, and in the reproductive, intracellular state, in which they behave as components of an integrated cellular system. Radiations, because of the discrete nature of their action, are more useful than chemicals in affecting free virus in such a way that, upon reintroduction into a host, the host-virus interaction will be abortive or variously modified; the results are very informative as to the biology of viruses. Moreover, it is possible by

\* This chapter has not been revised since being written in 1951. Valuable discussion and bibliography of more recent work are given by Pollard (1953).



means of radiation to reach a virus within the host cell and to obtain information as to its intracellular properties.

Although viruses do not constitute a homogeneous group that may a priori be expected to react to radiation in a uniform way, there does not appear to be any fundamental difference in the behavior of different viruses toward radiation, and most effects can be described under common headings for all viruses. The major differences reflect the different purposes for which radiation experiments with different viruses have been performed. In this chapter various types of radiation effects and applications of radiation analysis will be discussed, different viruses being used as examples rather than each virus being followed separately through the various approaches. It should be the goal of the radiobiologist to interpret the effects of radiation on viruses in terms of chemical alterations in nucleoproteins and other virus components. At this time, however, the radiation chemistry of large biological molecules is so poorly understood that a strictly chemical approach to the topic is precluded.

## 2. EFFECT OF RADIATIONS ON VIRUS INFECTIVITY

The most thoroughly investigated effect of radiation on viruses has been the loss of infectivity or "inactivation" of free virus particles when exposed to radiation. It must be recalled that "activity" of a virus can be defined as the ability to reproduce and to cause a detectable manifestation when introduced by a proper route into a suitable sensitive host. Quantitative studies are made possible by the relative accuracy of the titration methods for viruses; the amount of virus in different samples can be compared fairly accurately with a precision that may vary from 5 per cent in some instances to a factor of 2 or more in others. According to a majority of authors the results of virus titration give values proportional to the actual number of individual virus particles, each particle acting as one infectious unit with a probability that may be much lower than unity (Luria, 1940; Lauffer and Price, 1945). The results of titration can be used, however, to compare the active virus content of different samples (e.g., of an irradiated and a control suspension) even if infection requires the summation of the action of large numbers of virus particles rather than the reproduction of one successful particle, provided that a definite rule of proportionality exists between the amount of active virus introduced and the number or extent of the host manifestations. This is true, for example, for a method of titration that uses the incubation period or the time of death of an infected animal as related to the infecting dose (Bryan and Beard, 1939; Gard, 1943) rather than the counting of individual lesions or the dilution end point.

One important caution for the radiobiologist is the control of the mode of testing virus activity. This has become particularly important since

the recognition that bacteriophage inactivated by radiation can be reactivated in its host (Luria, 1947; Dulbecco, 1950); similar phenomena may occur for other viruses. In the case of bacteriophages, because of the possibility of reactivation, an inactive virus particle must be defined as one that is unable to parasitize a host cell or that, upon parasitizing a bacterial cell under conditions where no reactivation occurs, fails to give rise to the production of active bacteriophage (see Sect. 3-2).

A comparison of the titers of control and irradiated virus suspensions generally provides reproducible inactivation curves. In many cases the rate of inactivation of viruses by irradiation has been found to follow a simple exponential relation according to the equation

$$N = N_0 e^{-kD}, \quad (9-1)$$

where  $N_0$  = the titer in the unirradiated control,

$N$  = the titer in an irradiated sample,

$D$  = the dose of radiation, and

$k$  = a constant, characteristic for a given virus, a given radiation, and sometimes for a given mode of titration.

The dose for which  $N = N_0 e^{-1}$  is often called the "inactivation dose" and designated as  $D_{1/e}$  (Lea, 1946).

## 2-1. IONIZING RADIATIONS

X rays,  $\gamma$  rays,  $\alpha$  rays, electrons, neutrons, and deuterons have all been used to inactivate virus particles. It is now recognized that the inactivation of viruses by ionizing radiations may result from two major categories of effects: indirect and direct.

**2-1a. Indirect Effects.** These effects are mediated by toxic substances produced by radiation in dilute aqueous solutions. The toxic products are responsible for most of the inactivation observed, provided that sufficient amounts of protective substances which compete for the toxic products are not present. The current theories on the nature and chemical properties of the toxic substances produced by ionizing radiations in water and on the mode of their action are discussed by Dale and by Barron (in Vol. I of this series). As far as viruses are concerned, the indirect effect of X rays was first recognized on papilloma virus (Friedewald and Anderson, 1940, 1941), then on phage (Luria and Exner, 1941), and on plant viruses (Lea *et al.*, 1944). The effect manifests itself by a higher inactivation rate of viruses exposed to radiation in water or in saline solutions than when exposed either in crude suspensions containing large amounts of foreign substances or in the dry state. A variety of substances can act as protective agents. Proteins such as gelatin and egg or serum albumin are effective. Several substances of small molecular



weight, e.g., tryptophane, thiourea, and histidine, give complete protection of bacteriophage in very low concentrations (Latarjet and Ephrati, 1948; Watson, 1952). Ordinary bacteriological media such as beef broth are excellent protecting media.

Concentrated virus suspensions in nonprotective media are inactivated more slowly than dilute suspensions both because of the presence of impurities and because the virus itself may act as a protective agent. In some cases the inactivation rate does not increase with dilution beyond a certain point; the diluting medium itself may contain a small amount of protective substances. In very dilute suspensions there is also the possibility that some of the toxic agents produced by the radiation, such as free H and OH radicals, may recombine before having an opportunity to meet a virus particle and react chemically with it (Lea, 1946).

The rate of inactivation by the indirect effect of ionizing radiation varies from case to case. Deviations from the logarithmic relation Eq. (9-1), with the rate increasing with the dose, could be due to a need for cumulative damage of the virus (true multiple-hit effect), to a progressive accumulation of toxic products in the medium, or to destruction of protective substances by radiation itself. With purified tobacco mosaic virus, Lea *et al.* (1944) reported logarithmic rates of inactivation by X rays even for dilute suspensions of purified virus, but their data are not very satisfactory. For bacteriophages (Alper, 1948; Watson, 1952) the inactivation rate increases with time of exposure. This was interpreted by Alper (1948) as indicating progressive accumulation of toxic products in the medium. Watson (1952), however, was able to show that the increasing rate of inactivation was due almost exclusively to accumulation of damage in the phage particles. A phage sample in buffer received a small dose of X rays; most inactivation was due to indirect effect. The phage was then diluted in a completely protecting medium and later was again diluted in buffer and exposed to the same dose of X rays. The second exposure was much more effective than the first, as expected from the previously determined survival curve, although the second medium had not been irradiated previously.

Not all the indirect effect of X rays on viruses is exerted during actual exposure. There are also aftereffects of irradiation, mediated by relatively stable toxic products. If either water or buffer solutions are exposed to radiation, and virus is then introduced without appreciable amounts of protective substances, some inactivation will follow. In the case of bacteriophage (Watson, 1952) at least two agents are involved, one short-lived, detectable only during actual irradiation, and the other a long-lived one, which is quite stable at 5°C and is slowly inactivated at room temperature. Many of the effects of the latter can be duplicated by peroxides. The different nature of the two agents is evident from the different properties of phage inactivated by one or the other of them (see

Sect. 3-2). Protection against both agents is generally afforded by the same substances.

2-1b. *Direct Effect.* The inactivation of viruses by the indirect effect of ionizing radiations is mediated by chemicals produced in the medium. Its rate is affected by the temperature and by the distribution of dose in time, as expected from considerations of chemical kinetics. Because of its occurrence some questions have been raised as to the very existence of a direct effect exerted by the primary absorption of radiation energy within the physical domain of the virus particles. Yet such a direct effect is certainly present, and its analysis is possibly more revealing than that of the indirect effects, as far as the mechanism of biological effect of radiations is concerned. A *direct effect* of ionizing radiations is defined as a "nonprotectable" effect, i.e., an effect that cannot be eliminated by alterations of the medium. If the concentration of protective substances in the medium is increased beyond a certain level or if the virus is irradiated in the dry state, inactivation will proceed at a minimum rate, which cannot be further reduced (Luria and Exner, 1941; Lea, 1946). This residual inactivation is a function of only the total radiation dose and is not modified by changes in oxygen tension (Hewitt and Read, 1950), in temperature (Watson, 1950), or in the intensity of the radiation beam (Wollman *et al.*, 1940; Lea, 1946), thus exhibiting all the characteristics of photochemical reactions. The distinction between direct and indirect effects has recently been emphasized, at least in the case of bacteriophage, by the finding that the biological properties of phage particles inactivated by X rays in the presence of an excess of protective substances differ in many respects from those of particles inactivated by various types of indirect effects (Watson, 1952; see Sect. 3-2).<sup>1</sup>

The direct effect of ionizing radiation on viruses has been analyzed repeatedly in relation to the mechanism of radiation action not only on viruses but on genetic units and cells in general (Lea, 1946). In all well-investigated cases, inactivation of viruses proceeds according to Eq. (9-1). This indicates that one radiation "hit" inactivates a virus particle, i.e., a particle is inactivated by one successful act of absorption, without summation of individual effects.

Thus virus inactivation is a good test for further analysis based on the hit theory, and it has been employed widely in testing the validity of the

<sup>1</sup> Experiments by A. H. Doermann in 1951 (unpublished) show that addition of cysteine or BAL to a supposedly completely protecting medium such as nutrient broth can reduce the rate of bacteriophage inactivation by X rays by as much as a factor of 2. This important discovery, if confirmed, might force a revision of the definition of direct effect. More likely, it may be an indication that the direct effect, although direct in a geometric sense, i.e., exerted through acts of radiation absorption within the virus particle, is in part mediated through water bound around or within the virus in a way that permits agents such as cysteine or BAL to interact with the oxidizing products of water decomposition.



so-called "target theory," according to which the effective hits are those that occur within a specific physical domain which may coincide with all or parts of the biological object investigated. Information concerning the geometry of this domain is sought in the following way. An ideal "target" or "sensitive volume" is defined by the value of the constant  $k$  in Eq. (9-1):

$$k = \frac{1}{D_{1/e}}$$

If  $D$  is measured in acts of absorption per unit volume,  $k$  will have the dimensions of a volume (sensitive volume); if  $D$  is measured in number of paths of ionizing particles (such as protons) crossing a unit area, then  $k$  will have the dimension of an area, the "sensitive cross section." Clearly, the target or sensitive volume thus defined is not a priori identifiable with a physical portion of the biological object. Two extreme possibilities, and several intermediate ones, are conceivable:

Hypothesis 1. The target corresponds to a real volume, within which each hit is effective, whereas all hits without are ineffective.

Hypothesis 2. There is no such completely sensitive "real target"; the probability that a hit in a given unit volume is effective is distributed over a more or less large volume, which may even extend beyond the recognized boundaries of the organism.

In the case of viruses the recognition of the direct effect and its distinction from the indirect effects suggests that the probability that hits outside the physical borders of the virus particles are effective is not appreciably different from zero. The effectiveness of a hit within the particle, however, may be lower than unity and may vary from point to point, with a distribution  $p(x,y,z)$  over the volume of the particle. If  $c$  is the volume of the particle, Eq. (9-1) becomes

$$N = N_0 e^{-p(x,y,z) c D}.$$

If  $p(x,y,z) = P$  is constant, then

$$N = N_0 e^{-P c D}.$$

Hypothesis 1 would divide a virus into two parts, one with  $P = 1$  and the rest (if present) with  $P = 0$ . This point of view was vigorously defended by Lea (1946) who made extensive measurements of  $k$  for different biological effects of radiation and developed methods for analyzing the dependence of  $k$  on ionization density for various radiations. For the application of this type of analysis to viruses, the reader is referred to reviews by Lea (1946) and Bonet-Maury (1948). A brief summary will be sufficient here because the target theory is discussed by Fano (Vol. I of this series) and because, in the opinion of this writer, the information about viruses obtainable by this type of analysis is of limited value.

The first objective of the analysis has been to obtain values of  $k$  for different viruses, to compare them with the physical dimensions of the virus particles, and to obtain estimates of  $P$ . A major difficulty is the uncertainty as to what should be considered as the elementary act of absorption, i.e., the "hit." For example, if the elementary act is considered to be the production of one single ionization, a different value is obtained for  $k$  than if the elementary act is taken to be the production of a cluster of ionizations supposedly occurring so closely together as to produce only one hit. Moreover, an appreciable fraction of the energy absorbed from ionizing radiation will be dissipated in the form of excitations without ionization. Ultraviolet irradiation studies (see Sect. 2-2) have shown, however, that an excitation has a very low probability of producing virus inactivation. The part of energy dissipated in this way probably makes a minor contribution to the biological effect of ionizing radiation. In the case of radiations that produce dense columns of ionizations,  $k$  could be measured as the cross section for collision between the target and the ionization column. There is some uncertainty as to the size of the latter and even as to its approximate reducibility to a cylinder. It should be emphasized that these uncertainties are due to the limited amount of information available concerning the distribution of the energy released by ionizing radiation in liquids.

In spite of this, values of  $k$  for inactivation of several viruses are available for comparison with the known physical dimensions of the virus particles. A representative group of such data is presented in Table 9-1, from which the following facts emerge:

1. For a given virus, when the inactivation dose  $D_{1\%}$  for different radiation energies is measured in comparable units, based on ionizations per unit volume, there is a clear decrease of effectiveness per unit dose as the ionization density increases, as required by the target theory. Densely ionizing radiation should often produce more than one ionization (or cluster) within each target, with a resulting waste of ionizations. Lea (1946) proposed an elaborate method (the "associated volume method") to calculate the "true" size of the target (supposed to be spherical) from the dependence of  $k$  on ionization density. Target sizes obtained by this method are included in Table 9-1. For densely ionizing particles, Lea's method is approximately equivalent to measuring the cross section for collision between target and ionization column.

2. There is a certain degree of parallelism between particle size and radiation sensitivity for different viruses and for a given radiation, at least qualitatively and with a few exceptions. This is particularly evident for groups of agents presumably similar in kind, such as bacteriophages. This parallelism justifies the use of radiation data to estimate particle size by interpolation (Wollman and Lacassagne, 1940), a method that may still be of use when a virus cannot be purified enough for electron



TABLE 9-1

Virus	Radiation	Inactivation dose* ( $D_{1/e}$ ), r	Refer- ence†	Diam- eter of spheri- cal target per ion pair, m $\mu$	Diam- eter of spheri- cal target accord- ing to Lea's "associ- ated volume meth- od,"‡ m $\mu$	Particle diameter§ (or linear dimensions if not spherical), m $\mu$
Bacteriophages:						
S13.....	$\gamma$	$5.8 \times 10^5$	4	12	15.5	16-20 (filtration, centrifugation; shape unknown)
	X rays, 1.5 A	$9.9 \times 10^5$	4	10	15.9	
	$\alpha$ , 4 Mev	$3.5 \times 10^6$	4	7	16.3	
$\phi$ X-174.....	X rays, 0.9 A	$6.8 \times 10^5$	1	12	17	15-20 (filtration, centrifugation; shape unknown)
	$\alpha$ , 6.5 Mev	$4.9 \times 10^6$	1	6	13	
C36.....	$\gamma$	$2.1 \times 10^5$	4	18	21.5	42 (filtration; shape unknown)
	X rays, 1.5 A	$4.3 \times 10^5$	4	14	22.3	
	$\alpha$ , 4 Mev	$9.4 \times 10^5$	4	10	33	
T1 (P28)....	X rays, <0.1 A	$9 \times 10^4$	5	23	30	Head, 50; tail, 15 $\times$ 200 (tadpole-shaped; electron microscope)
	Deuterons:					
	3.5 Mev	$3.2 \times 10^5$	6	16	25-30	
	2.5 Mev	$3.9 \times 10^5$	6	15		
	1.5 Mev	$4.9 \times 10^5$	6	14		
Staph. K....	$\gamma$	$7.9 \times 10^4$	4	24	31	60-100 (filtration)
	X rays, <0.1 A	$4.5 \times 10^4$	5	30	40	
	X rays, 1.5 A	$1.1 \times 10^5$	4	21	40	
	$\alpha$ , 4 Mev	$4.5 \times 10^5$	4	14	50	
C16 (T2,T4,T6)	X rays, <0.1 A	$4 \times 10^4$	5	30	42	Head, 60 $\times$ 80; tail, 20 $\times$ 120 (tadpole-shaped; electron microscope)
	X rays, 0.1 A	$3.5 \times 10^4$	8	31	42	
	X rays, 0.15 A	$4 \times 10^4$	9	30	40	
	X rays, 0.7 A	$4.5 \times 10^4$	9	29	58	
	$\alpha$ , 6.5 Mev	$2.1 \times 10^5$	9	18	70	
Vaccinia.....	$\gamma$	$8 \times 10^4$	3	24	31	210 $\times$ 260 (brick-shaped; electron microscope)
	X rays, 0.9 A	$3.5 \times 10^5$	1	22	41	
	X rays, 1.5 A	$1 \times 10^5$	3	18	70	
	$\alpha$ , 4 Mev	$2.1 \times 10^5$	3	16	23	
	$\alpha$ , 6.5 Mev	$3.5 \times 10^4$	1	32	175	
Herpes.....	$\alpha$ , 6.5 Mev	$3.5 \times 10^4$	1	32	175	265 $\times$ 300 (brick-shaped; electron microscope)
Foot and mouth	X ray, 0.9 A	$2.8 \times 10^5$	1	17	27	15-30 (filtration, centrifugation; shape unknown)
	$\alpha$ , 6.5 Mev	$1.3 \times 10^6$	1	10	23	
Rabbit papil- loma.....	X rays, 0.1 A	$4.4 \times 10^4$	7	29	40	66 (centrifugation)
Tobacco necrosis	$\gamma$	$6.7 \times 10^5$	2	12	14	27.5 (electron microscope)
	X rays, 1.5 A	$9.4 \times 10^5$	2	10	16	
	X rays, 8.3 A	$5.15 \times 10^6$	2	6	12	
Tomato bushy stunt	$\gamma$	$4.5 \times 10^5$	2	14	17	25.5 (electron microscope)
	X rays, 1.5 A	$6.2 \times 10^5$	2	12	19	(37) (hydrated)
	X rays, 8.3 A	$3.1 \times 10^6$	2	7	14	
	$\alpha$ , 4 Mev	$2.6 \times 10^6$	2	8	22	
Tobacco mosaic	$\gamma$	$3.7 \times 10^5$	2	15	18	15 $\times$ 280 (rod-shaped; electron microscope)
	X rays, 1.5 A	$4.3 \times 10^5$	2	14	22	
	X rays, 8.3 A	$1.5 \times 10^6$	2	9	19	
	$\alpha$ , 4 Mev	$1.9 \times 10^6$	2	9	26	

\* When the inactivation dose was not given in roentgens by the authors, it was calculated on the assumption that 1 r corresponded to  $1.7 \times 10^{12}$  ion pairs per cubic centimeter for  $\gamma$  and X rays and to  $1.9 \times 10^{12}$  for  $\alpha$  rays and deuterons.

† References: (1) Bonet-Maury, 1948. (2) Lea, 1946. (3) Lea and Salaman, 1942. (4) Lea and Salaman, 1946. (5) Luria and Exner, 1941. (6) Pollard and Forro, 1949. (7) Syverton, Berry, and Warren, 1941. (8) Watson, 1950. (9) Wollman *et al.*, 1940.

‡ The values are either taken from Lea (1946) or read from Fig. 8 in Lea (1946). Since Lea assumed a value of 1.35 for the density of viruses, this value was adopted throughout, although for viruses irradiated in liquid a value of 1.13 would be a more likely estimate. In no case would such a difference affect the calculated diameters, which at any rate are simply rough estimates, by more than 10 per cent.

§ The particle sizes obtained by filtration or centrifugation correspond to "wet" sizes; those by electron microscopy are "dry" sizes.

microscopy or ultracentrifugal analysis and when calibrated ultrafilters are unavailable.

3. There is a progressively increasing discrepancy between particle volume and target volume, proceeding from small to large viruses, particularly with sparsely ionizing radiation. Radiation sensitivity increases more slowly than virus size.<sup>2</sup> This is where the weakness of the target theory becomes evident.

Lea (1946) favors the hypothesis that the target measures a true physical volume within which each ionization is effective. The smallest viruses would be fully sensitive, whereas the particles of the larger viruses would contain both a radiation-sensitive (genetic) portion and a nonsensitive (nongenetic) portion. This analysis is carried further with the assumption that the sensitive portion always consists of one or more spheres. If the dependence on ionization density indicates that the effectiveness does not decrease fast enough with increasing density, it is postulated that the target consists of several spheres (a less wasteful arrangement for overlapping ionizations). Moreover, each sphere is compared to a gene, whose lethal mutation results from a hit, and an estimate of the number of genes per virus particle is derived; for example, vaccinia virus would contain about 100 genes, and a large phage would contain 10 or 20 (Lea and Salaman, 1946). This type of analysis hardly seems justified.

In the first place, there are neither radiochemical nor genetic reasons to postulate a differentiation of biological materials into either indispensable and fully sensitive to radiation or fully dispensable. For viruses, it seems likely, in view of the complicated stages of interaction between virus and host, that many portions of the virus may be altered by radiation in such a way as to prevent reproduction. For example, surface groups may be involved in adsorption onto the host cells; other groups may be operative in replication; and others in removing inhibitors. The probability of effective damage by one ionization (or cluster) may be different from one portion of a virus to another. For example, as mentioned later (see Sect. 3-2), phage particles inactivated by X rays (direct effect) fall into two categories, some capable and some incapable of killing the bacterial host (Watson, 1950). The two types are probably damaged in different parts or in different ways.

In the second place, even if there were a target within which an act of absorption was always effective, there would be no reason for its geometric interpretation as a sum of spheres. The observed dependence of radia-

<sup>2</sup> It has been stated that the discrepancies almost disappear for  $\alpha$  particles (Bonet-Maury, 1947, 1948); this seems doubtful, however.  $\alpha$ -ray data include disturbing contradictions such as a great disparity in sensitivity of vaccinia virus as measured by different authors (Lea and Salaman, 1942; Bonet-Maury, 1947) and a target size reported for poliomyelitis virus (Lansing strain) much larger than the generally accepted virus size (Bonet-Maury, 1948).



tion efficiency on ionization density could result from any geometric deviation from the "single spherical target" model.<sup>3</sup>

A study of the inactivation of a bacteriophage by deuteron beams of different energy (Pollard and Forro, 1949) showed a dependence of effectiveness on the energy of the beam. This was interpreted at first as being due to a difference in the effective diameter of the ionization column because of ultraviolet emission by excited atoms near the target but outside it. This was clearly incompatible with the known low quantum yield for ultraviolet inactivation of phage, and the deuteron data have been reinterpreted (Pollard, 1951) by a method substantially equivalent to the associated volume method of Lea (1946) and open to similar criticisms.

In conclusion, it may be said that radiation studies on viruses based on the target theory have not yet provided any basic information on the nature and structure of viruses, especially because not enough attention has been paid to the interaction of an irradiated virus with its host. A virus particle is defined as inactive when it has become unable to produce active replicas of itself. This failure of reproduction may be caused either by inability of the virus to attach itself to a host or to a susceptible cell, by inability to penetrate and invade the host, or by inability to carry out any one of the probably numerous steps intervening between infection and production of new active virus in an infected cell.<sup>4</sup> Different portions of a virus particle may be functioning in each of these processes. Chemical changes may be produced in a virus particle by radiation or other means without the particle registering as inactive, either because the damaged portion is not essential for reproduction or because that portion, although used in reproduction, may be replaceable by other portions. For example, suppose that a virus particle has a discrete number of different surface areas, *A*, *B*, *C*, . . . , any one of which can act to the exclusion of others as the "receptor" involved both in adsorption of the particle on a susceptible cell and in the following penetration, and suppose that, if receptor *A* is utilized for absorption, the same receptor *A* will also control penetration. If receptor *A* is damaged by a radiation hit in such a way that it

<sup>3</sup> A similar criticism can be made of an attempt (Bonet-Maury, 1948) to interpret the supposedly lower sensitivity of vaccinia virus to X rays than to  $\alpha$  particles (an unconfirmed observation; see Lea and Salaman, 1942) by assuming that the virus particle consists of an agglomeration of individual units, each of which must be inactivated for suppression of infectivity. The  $\alpha$  rays supposedly would inactivate all the units in the agglomerate by energy spread. This hypothesis would require that each particle break apart into single units before infection; otherwise, the X-ray inactivation curve would be of the multiple-hit type. There is no evidence for such a structure of the vaccinia virus particle.

<sup>4</sup> In the case of viruses acting on multicellular organisms, if activity is detected by appearance of multicellular foci of infection or of general reactions, a virus may be considered inactive if it is unable to reproduce sufficiently to overcome the host defenses.

can still function in adsorption but not in penetration, reproduction will be blocked whenever receptor  $A$  is utilized, and the probability that inactivation results from such a hit is the inverse of the number of areas  $A, B, C, \dots$  present on the virus surface. Considerations of this type underline the naïveté of the target theory in its narrow form, as applied to the analysis of virus inactivation.

An interesting result has recently been obtained (Hershey *et al.*, 1951) from a study of the spontaneous inactivation of bacteriophage that contains relatively large amounts of radioactive phosphorus. Bacteriophage particles containing up to 100 or more  $P^{32}$  atoms (out of a total of approximately 500,000 phosphorus atoms in nucleotides) show a definite instability, with a one-hit type of activity decay. On the average, one particle is inactivated for every ten  $P^{32}$  disintegrations. The inactivation is apparently not due to the emission of  $\beta$  rays, but to the nuclear event itself. This result suggests either that only 10 per cent of the phosphorus atoms of a phage particle are necessary for infectivity, the others being dispensable, or that, when a phosphorus nucleus disintegrates, there is an average probability 0.10 that this change will result in inactivation.

Some authors (see Riehl *et al.*, 1941) have discussed the problem of how a hit in any one point of a large physical volume can produce inactivation of a virus (or mutation of a gene) and have speculated on the possible need and mechanism for energy migration within a large biological molecule to a specific site of action. Such an approach has not led very far, however, since little is known about such energy-migration mechanisms. The need to invoke their intervention in virus inactivation is not apparent.

## 2-2. ULTRAVIOLET RADIATION

Viruses have generally been exposed to ultraviolet radiation either in stirred suspensions or in thin layers in order to avoid or equalize the screening effect of impurities. A continuous-flow technique has also been described (Levinson *et al.*, 1944). The possibility that in such experiments some indirect effects of radiations may be observed has often been neglected since the doses of ultraviolet radiation needed for inactivation of viruses do not seem to produce appreciable amounts of toxic substances in water. Toxic products might, however, originate from impurities.

For most viruses the proportion of active virus has been reported to decrease exponentially with the dose according to Eq. (9-1), the total dose (intensity times time) being the relevant variable (Hollaender and Duggar, 1936; Price and Gowen, 1937; Taylor *et al.*, 1941; Latarjet and Wahl, 1945; Oster and McLaren, 1950; Fluke and Pollard, 1949). One quantum is apparently the effective hit. Recent data on bacteriophage, for which the precise titration method makes it possible to obtain more accurate inactivation curves, indicate deviations from the simple logarithmic relation. Some phages (T2, T4, T6) exhibit a slow initial rate of



inactivation for very small doses (about  $10 \text{ ergs} \times \text{mm}^{-2}$  for 2537 Å), soon changing to a logarithmic rate as the dose increases (Benzer *et al.*, 1950). The reason for this behavior is obscure; it does not seem to be due to the presence of aggregates of virus particles. Other phages (e.g., T1, T7) show an initial logarithmic rate with a break to a slower rate for survivals lower than  $10^{-2}$ . The more resistant fraction of virus is not genetically different; it is possible that it is combined with screening materials. Complications of this kind make calculations of inactivation rates and of quantum yields somewhat questionable.

Action spectra have been reported for several viruses (Rivers and Gates, 1928; Sturm *et al.*, 1932; Gates, 1934; Hollaender and Duggar, 1936; Hollaender and Oliphant, 1944). Comparisons were generally made only for incident energies, however, by plotting the inverse of the incident dose required to produce a constant amount of inactivation versus the wave length. For most viruses the graph resembles the absorption curve of nucleic acids with a minimum at 2400 Å, a maximum around 2600 Å, and very low effectiveness beyond 3000 Å. For some viruses, however, the maximum and minimum at 2600 and 2400 Å, respectively, are much less pronounced than for other viruses. There is no clear correlation between total nucleic acid content and type of action spectrum since vaccinia virus and tobacco mosaic virus have approximately the same nucleic acid content (in percentage of dry weight), yet give different action spectra. It has been pointed out that the viruses, whose action spectra are less similar to the absorption spectrum of nucleic acid, supposedly contain the ribose instead of the deoxyribose type (Hollaender, 1946).

Although these results indicate that, at least in most cases, a large proportion of the effective radiation is absorbed by the nucleic acid of the virus particles, they do not indicate the relative effectiveness of quanta absorbed by different virus components. If the part of a radiation absorbed by nucleic acid and that absorbed by other components, e.g., proteins, were equally effective in producing inactivation, the greater absorption coefficient of the nucleic acids for most ultraviolet wave lengths would cause them to appear as the main contributors to the effective absorption whenever they are present in the amounts found in many viruses (5–40 per cent of the dry weight).

More information could be gained from action-spectrum studies based on measurements not of incident radiation energy but of actual quantum yield. Absorption measurements on purified virus preparations are easily feasible, yet surprisingly few data on quantum yield for virus inactivation have been reported. In most cases they are for one wave length only, the 2537 Å line of mercury. One difficulty, of course, is that the actual virus content of a preparation, in terms of particles per milliliter, is seldom accurately known. For tobacco mosaic virus and 2537 Å the values of  $2.6 \times 10^{-5}$  (Uber, 1941) and  $4.3 \times 10^{-5}$  (Oster and McLaren, 1950) have been reported for the quantum yield, the latter value being probably more

accurate. For phage T2, the quantum yield (2537 Å) is about  $10^{-4}$  (M. R. Zelle, personal communication), assuming a one-hit mechanism in spite of the deviation from the logarithmic inactivation rate. These quantum yields are much lower than those reported for several chemical changes in simple organic compounds, including nucleic acid constituents (0.01–0.1). Most absorption takes place in the nucleic acid component of the virus. Unless absorption in this component happens to be much less effective than that in some other components (which is not supported by our knowledge of the action spectra), we are led to suppose that a virus can withstand an appreciable amount of chemical change in its nucleic acid moiety without being inactivated. This is in agreement with the results of experiments on phage inactivation following radioactive decay of its  $P^{32}$  atoms (Hershey *et al.*, 1951; see Sect. 2-1), and the same considerations apply to both instances.

The ultraviolet sensitivity of several viruses of a certain group, such as bacteriophages, roughly parallels the particle size when the doses are measured in incident energy (Luria and Dulbecco, 1949). This probably reflects in part the greater cross section of larger viruses and suggests that the quantum yields for inactivation may be of the same order. Bacteriophages T2, T4, and T6 have equal size and morphology, yet T4 is twice as resistant to ultraviolet (2537 Å) as T2 or T6. It is not yet clear whether this difference is due to a lower nucleic acid content or to a lower quantum yield. The radiation resistance of T4 becomes associated with some of the distinctive characteristics of T2 or T6 in type-hybrid phages produced by mixed infection (Luria, 1949). This makes it possible to investigate the determination of the ultraviolet sensitivity of a group of viruses by genetic means.

### 2-3. VISIBLE LIGHT

Wahl and collaborators (Wahl, 1946; Wahl and Latarjet, 1947) found that several bacteriophages are inactivated at an appreciable rate when exposed to visible light. The action spectrum has a maximum in the near-ultraviolet and violet regions and a limit of effectiveness in the green region of the spectrum. Yellow and red light are ineffective. This might indicate that the viruses contain a pigment with a maximum of absorption or of photochemical yield for the long ultraviolet radiation. It is unknown whether this pigment plays any role in the photoreactivation phenomenon (see Chap. 12 of this volume). It has also been suggested that the inactivation may be due to a photodynamic action mediated by components of the medium (Dulbecco, personal communication).

## 3. DIFFERENTIAL EFFECT OF RADIATIONS ON VARIOUS PROPERTIES OF VIRUSES

The loss of ability to reproduce is only one of the alterations that may be produced in a virus particle. Since inactivation results from suppres-



sion of any one of the steps needed for successful infection and virus reproduction, it is often more easily affected than any other recognizable property of the virus particles. Changes produced by radiation, other than inactivation, may be even more interesting than inactivation itself since they may reveal new properties of the virus particles and their dependence on the integrity of specific virus functions.

Only very large radiation doses cause actual disintegration of the particles. In this section are considered, first, nonlethal effects of radiation, i.e., changes recognizable in virus particles that survive irradiation; then a series of changes recognizable in inactive particles.

### 3-1. NONLETHAL EFFECTS

Nonlethal effects are recognized as alterations in the properties of those virus particles that survive exposure to radiation; some alterations are nonhereditary, others are transmitted to the progeny. Among the former may be mentioned a delay in reproduction of bacteriophage particles that survive ultraviolet irradiation, as evidenced by an increase in the latent period between infection of a bacterium and its lysis with liberation of new virus (Luria, 1944). The new virus gives a normal growth cycle; the reproductive delay, then, persists for only one cycle of intracellular reproduction. Another nonlethal effect consists in a slower adsorption by bacteria of phage surviving exposure to X rays under conditions where indirect effects are prevalent (Watson, 1952); there is probably a surface alteration of the phage by toxic substances produced by X rays in the medium.

A more important group of nonlethal effects of radiation on viruses is the induction of phenotypic mutations, a field as yet insufficiently investigated. Exposure of tobacco mosaic virus to X rays has been reported to produce mutations both from wild type to aucuba and back (Gowen, 1941). The data indicate that the probability of inducing a mutation is about one one-thousandth that of inactivating a virus particle. A report is available on mutation induced in tobacco mosaic virus by irradiation of virus-infected leaves (Pfankuch *et al.*, 1940).

With bacteriophage T2, Latarjet (1949) has reported that, following ultraviolet irradiation of infected bacteria, there is an increase in the proportion of bacteria that liberate phage mutants T2h.

### 3-2. SEPARATION OF PROPERTIES OF INACTIVE VIRUS PARTICLES

The detection of the effect of radiations on different properties of viruses depends on the number of properties recognizable by the limited mode of analysis. With viruses such as bacteriophages and influenza viruses, several properties can be separated by increasing doses of radiation or by different types of radiations. Some groups of properties, however, are always lost simultaneously. When the properties studied represent

recognizable events in the interaction of a virus with the host cell, the simultaneous loss of two properties may be taken as an indication that the corresponding events result from the same step in interaction. Thus, successive steps in host-virus interaction can be traced by the analysis of the residual properties of virus particles exposed to different radiations under different conditions. As an example, this type of analysis as carried out for bacteriophages T2, T4, and T6 active on the common host *Escherichia coli* B will be described.

The major phases of interaction between these viruses and their common host are fairly well known (for reviews, see Delbrück, 1942; Cohen, 1949; Luria, 1950; Benzer *et al.*, 1950). One or more active particles of bacteriophage become adsorbed by the susceptible bacterium; the adsorption is irreversible under the usual environmental conditions. Reproduction of the bacterial cell is stopped, and there is complete suppression of the synthesis of the specific components of bacteria, in particular, of bacterial enzymes. There occurs a quick and profound change in the cytologically recognizable nuclear apparatus of the bacterial cell, which in fixed preparations appears to be disrupted and is later replaced by a fine, granular material giving the cytochemical reactions of deoxyribonucleate and probably representing the new virus. All synthetic processes in the infected cell are directed toward the synthesis of bacteriophage components—phage protein and phage nucleic acid—through the activity of preexisting bacterial enzymes. After a rather precisely defined latent period, during which the synthesis of phage components is followed by the appearance of large numbers of new phage particles, the bacterial cell is lysed and releases the new phage into the medium.

The outcome of the infection also depends on the number and genetic constitution of the infecting particles. If too many particles are present, there may occur a "lysis from without," apparently resulting from a massive damage to the bacterial surface. This type of lysis takes place without phage reproduction and without disruption of the bacterial nuclei. The T-even bacteriophages, in their wild types, also exhibit a phenomenon of "lysis inhibition," i.e., a delay in lysis if two or more particles infect a bacterium at an interval of several minutes.

Let us see what happens if active bacteriophage particles are replaced by particles inactivated by exposure to radiation.

Phage particles inactivated by moderate doses of ultraviolet radiation ( $N/N_0 > e^{-30}$ , by extrapolation), if tested under conditions where no reactivation occurs (see Sect. 4-2), are still capable of being adsorbed by bacteria and of killing the bacterial cell (Luria and Delbrück, 1942). Bacterial nuclei are disrupted and bacterial syntheses are suppressed, but no synthesis of phage components takes place. Lysis and liberation of active bacteriophage are absent; even if the infected cells are artificially broken, no active bacteriophage is extracted (Luria and Human, 1950).



Thus ultraviolet irradiation separates the early phases of infection from the later ones; it provides evidence that bacterial killing occurs through the disrupting action of the infecting phage and does not require its reproduction. With other ultraviolet-inactivated phages (e.g., T1 and T7) bacterial infection is followed by an increase of material that reacts cytochemically like deoxyribonucleic acid, but no active phage can be recovered from the bacteria.

For X-ray inactivation, it is necessary to distinguish between direct and indirect effects (see Sect. 2-1). Bacteriophage particles inactivated by the direct effects of X rays (Watson, 1950) are normally adsorbed by host bacteria, but their bacteria-killing ability is often lost. The fraction of "killing" particles diminishes logarithmically with the X-ray dose, with a slope approximately one-third the slope of the inactivation curve. The killing particles affect the bacteria in the same way as does ultraviolet-inactivated phage. All adsorbable phage particles, whether killers or not, retain both the "lysis-inhibiting" property and the ability to produce "lysis from without." These effects, then, require only the changes brought about by phage adsorption, without further intromission of the virus particle into the economy of the host cell. Thus the comparison between active phage particles and particles inactivated by ultraviolet and by X rays permits the distinguishing, in the early preproductive phases of host-virus interaction, of two stages—one of "adsorption" and one of "invasion." The latter involves the disruption of that part of the bacterial machinery that impresses on the newly synthesized material the specificity of bacterial protoplasm. Interference phenomena (see Sect. 4-1) require particles capable of invasion; they are produced only by particles that can kill bacteria.

Phage particles inactivated by the indirect effect of X rays (exposure in the absence of protective substances; Watson, 1952) exhibit a greatly reduced rate of adsorption onto the host cells, which hinders the analysis of those phage properties that manifest themselves in later stages of the host-virus interaction. This suppression of adsorption results only from exposure to the short-lived toxic agent present during actual irradiation in water. Phage particles inactivated by introduction into a freshly irradiated medium give a completely different picture; they are readily adsorbed and retain their killing ability, with all the properties that attend this. No interpretation in chemical terms of the effects of indirect irradiation on various phage properties is available; differential effects of chemical poisons on different parts of virus particles are clearly to be expected.

Generally, inactive virus particles that are still adsorbable by the bacterial cells are not physically disintegrated and can still be recognized, e.g., in electron micrographs. Very large doses of ultraviolet radiation disrupt the complex morphological structure of some bacteriophages. Upon disruption, some of the large coli bacteriophages release part of their nucleic

acid (Dulbecco, 1950) and also liberate an agent, smaller than the virus particles and separable by differential centrifugation, which produces lysis of the susceptible bacteria (Anderson, 1945). This lytic agent may or may not be implicated in the normal lysis of bacteria infected with active phage; the possibility of liberating active principles from virus particles by means of radiation is, at any rate, suggestive of a new approach to virus research.

The situation described for the phages of the T group is by no means unique; at least for influenza viruses, similar observations have been made (Henle and Henle, 1947). Exposure to ultraviolet for progressively longer periods of time eliminates, one after the other, all the properties of the virus that can be studied. Reproductive ability disappears first, followed by toxicity, which, according to Schlesinger (1950a), is a manifestation of an abortive infection in cells incapable of supporting full reproduction of the virus. The ability of the virus to interfere with the reproduction of another virus disappears next, followed by the immunizing capacity for a susceptible host (which may have to do with both antigenicity and interfering ability). Hemagglutination—that is, the ability to agglutinate red blood cells—is much more resistant and disappears only after doses of radiation which probably disrupt the virus particle. Complement-fixing antigens, mainly present in crude virus preparations in the form of small “soluble” antigens, are greatly resistant to irradiation. It is interesting that hemagglutination and complement fixation should be the two most resistant properties of the influenza virus since both of them can be found separated from virus activity in the course of normal growth (Hoyle, 1948; Henle and Henle, 1949) and may be in the form of immature elements of greater ultraviolet resistance.

It may be noted that, to an inactive particle of influenza virus, radiation can leave both the ability to agglutinate red blood cells and the ability to be eluted from them enzymically, whereas heat, for example, preserves the ability to agglutinate red blood cells but suppresses the enzymatic elution.

The separation of infectivity from the antigenic properties of a virus by radiation is of fairly general observation. It has been proved for phages, for plant viruses (which retain enough of their integrity to form the same crystals or paracrystals as their active counterparts; see Bawden, 1950), and for a series of animal viruses. The persistence of serological properties, however, may be limited to the effect of ultraviolet or of X rays acting directly. In the case of papilloma virus, the indirect effect of X rays gives a closer parallelism between the destruction of infectivity and that of complement fixation than does the direct effect of X rays (Friedewald and Anderson, 1941).

Because of the persistence of its antibody-stimulating ability, virus which has been inactivated by radiation, in spite of some observation to



the contrary, is now considered a rather good source of vaccines (Webster and Casals, 1942; Levinson *et al.*, 1944; Milzer *et al.*, 1944; Milzer and Levinson, 1949). As is the rule with inactive viruses, large amounts of irradiated virus must be used in vaccination since there is no increase in antigen by multiplication of virus in the host. It is possible that a certain role in the immunity phenomena observed with inactive virus vaccines may be played by the interference phenomena discussed in the next section.

#### 4. IRRADIATED VIRUS IN MULTIPLE AND MIXED INFECTION

##### 4-1. INTERFERENCE PHENOMENA

Under interference phenomena is included a complex group of phenomena involving an alteration in the growth or manifestations of a virus due to the presence in the same host of more virus of the same or another type. The virus particles do not interact among themselves *in vitro*, and the interference phenomena are strictly cellular. Only with bacteriophage, however, have interference phenomena been analyzed at the cellular level (Delbrück, 1950).

Mixed infection of a common host with two unrelated bacteriophages results in mutual exclusion, only one virus type reproducing in any one given cell. The excluded virus may exert a depressor effect on the yield of winning virus. These exclusion phenomena are not exerted at adsorption but take place intracellularly. Related viruses give incomplete exclusion, which becomes less and less evident as the viruses become more closely related; particles of two virus strains differing by one mutation only do not exclude one another. Whenever exclusion fails, the total yield of virus per cell is lower than the sum of the yields that each virus would produce by itself; the two viruses share the maximum potential yield per cell.

With irradiated phages the following rule is fairly well established: Whenever a phage particle, after exposure to ultraviolet or X rays, can still invade and kill the cell, it retains the exclusion power it had when active; particles that are adsorbed but do not kill the host do not produce exclusion (Luria and Delbrück, 1942; Watson, 1950). It is not known whether an ultraviolet- or an X-ray-inactivated virus particle, if excluded, can still exert a depressor effect on the yield of an active, heterologous, excluding virus. Irradiated interfering phage excludes homologous active phage if it reaches the bacterium several minutes earlier; otherwise, exclusion fails, and the yield of active phage is normal (Luria and Dulbecco, 1949).

It is evident then that the interfering ability of inactivated phages is related to their ability to kill the bacterial host. If, as seems likely, the latter process results from the virus taking over and redirecting the syn-

thetic machinery of the host, interference is probably also a manifestation of the appropriation by one virus of the directive pattern of specificity to the exclusion of another virus. It is interesting in this connection that "lysogenic" bacteria, which carry a phage in a form that does not interfere with bacterial life, can be infected and lysed by other, unrelated phages and can liberate them normally.

In the case of animal and plant viruses, interference phenomena have generally been studied only in their mass manifestations when a tissue or a whole organism is exposed to two viruses in succession or simultaneously. It is difficult therefore to interpret interference in terms of cellular events. For animal viruses, interference phenomena have been discussed by Henle (1950) and Schlesinger (1950b). Interference may occur between related or unrelated viruses, but not all unrelated viruses interfere with one another, and two viruses can often be shown to multiply in the same cell. Whenever there is interference between two active viruses, it is also observed with virus inactivated by ultraviolet radiation; other radiations have hardly been studied in this connection. With influenza viruses in the allantoic cavity of the chick embryo, it has been shown (Henle and Henle, 1943, 1945; Ziegler *et al.*, 1944) that a large amount of a virus, e.g., influenza type A, after inactivation by ultraviolet radiation can prevent reproduction either of homologous or of heterologous active virus, e.g., influenza type B. This interference was at first attributed to suppression of virus adsorption because the irradiated virus destroyed the virus receptors on the allantoic cells. It is now known, however, that interference may occur with amounts of virus that do not prevent adsorption and also by introduction of the interfering virus after the first virus has been adsorbed (Henle, 1950). In the case of bacteriophage, as well as influenza virus, interference probably takes place at the level of the reproductive process. That a blockade of the synthetic machinery is involved, rather than a competition for building blocks, is suggested by the fact the inactive virus, although unable to reproduce, retains the interfering ability.

#### 4-2. REACTIVATION PHENOMENA

Reactivation phenomena have been reported only with bacteriophages, but the possibility of their occurrence in other viruses should be explored. Bacteriophages exposed to radiation give different activity titers according to the conditions of titration. Two factors have been found relevant: The number of irradiated particles adsorbed per bacterium (Luria, 1947) and the exposure of the infected bacteria to light of certain wave lengths (Dulbecco, 1950). No reactivation effect has been observed following treatments of irradiated phage before adsorption to the host bacterium. Reactivation of phages by light ("photoreactivation") is discussed in detail in Chap. 12 of this volume.

4-2a. *Multiplicity Reactivation.* In phage titration the phage must be



mixed with sensitive bacteria. For irradiated phage the residual titer is minimum when the phage is exposed to such an excess of bacteria that the great majority of the infected bacteria receive only one phage particle. Under these conditions, it is possible to distinguish a fraction of "residual active particles" and one of "inactive particles." The usual inactivation curves are obtained in this manner. For some phages and for some types of radiation, there is an apparent increase in activity under conditions of "multiple infection" of bacteria with phage. This multiplicity reactivation has been observed with the coli phages T1, T2, T4, T5, and T6 after ultraviolet irradiation (Luria and Dulbecco, 1949) and with T2, T4, and T6 also after exposure to X rays (Watson, 1950). To participate in reactivation, an irradiated particle must retain its ability to kill the host (see Sect. 3-2).

TABLE 9-2. THE BASIC OBSERVATION IN MULTIPLICITY REACTIVATION OF BACTERIOPHAGE

(Modified from Luria and Dulbecco, 1949, Table 2.)

Phage: T6r,  $1.5 \times 10^{10}$  units/ml exposed for 20 sec to ultraviolet germicidal lamp,

General Electric Company, 15 watts, at 50 cm; 7 ergs/mm<sup>2</sup>/sec

Bacteria: *E. coli* B,  $2 \times 10^9$  cells/ml = B

Platings: 0.05 ml of phage dilution plus 0.2 ml of B per plate

Mixture No.	Procedure	Dilution of phage when first mixed with B	Total dilution of original phage in suspension from which samples were plated	Plaque count (sum of two plates)
1	0.1 ml T6r → 0.9 ml B; kept 10 min at 37°C; diluted 1 to 10 <sup>3</sup> , 0.05 ml plated	1/10	1/10 <sup>4</sup>	1318
2	0.1 ml (T6r 1/10) → 0.9 ml B; kept 10 min at 37°C; diluted 1 to 10 <sup>2</sup> , 0.05 ml plated	1/10 <sup>2</sup>	1/10 <sup>4</sup>	474
3	0.1 ml (T6r 1/10 <sup>3</sup> ) → 0.9 ml B; kept 10 min at 37°C; diluted 1 to 10, 0.05 ml plated	1/10 <sup>4</sup>	1/10 <sup>4</sup>	250
4	0.05 ml (T6r 1/10 <sup>4</sup> ) plated	< 1/10 <sup>5</sup> (on plate)	1/10 <sup>4</sup>	57

The basic observation is illustrated in Table 9-2 and consists in the fact that the same amount of irradiated phage gives a higher activity titer (number of lytic areas or "plaques" on a solid layer of sensitive bacteria) if the bacteria have been allowed to adsorb the phage from a more concentrated phage suspension. The effect is not caused by exposure of the infected bacteria to some factor other than phage present in crude concentrated phage preparations since it occurs equally well with purified phage.

It must be remembered that, in the type of titration employed, only bacteria which, after receiving phage particles, liberate active phage are measured. Only these bacteria are lysed; infected bacteria that fail to liberate active phage die unlysed.

If the number of bacteria that liberate active phage is determined and the number of residual active phage particles is subtracted from it, the number of bacteria in which inactive phage was reactivated is obtained. This is never higher than the number of cells that receive two or more inactive particles. For small doses of ultraviolet radiations and for high multiplicities of infection, the two become approximately equal. Thus, reactivation is due to intracellular interaction between phage particles which, if adsorbed on separate bacteria, would have registered as inactive.

The interpretation of the mechanism of "multiplicity reactivation" is at this time obscure. The theory originally proposed for its interpretation (Luria, 1947; Luria and Dulbecco, 1949) is undergoing revisions. When the phenomenon was first recognized, it was quickly discovered that reactivation occurs not only among particles of the same phage but also among particles of two related phages. More especially, it occurs among particles of phages T2, T4, and T6. These exhibit the remarkable phenomenon of genetic recombination, in which mixed infection with two different phages results in the production of "hybrid forms," deriving some of their properties from one phage, some from the other (Delbrück and Bailey, 1946; Hershey and Rotman, 1948, 1949).

This observation suggested a similarity of mechanism between recombination and reactivation, and the hypothesis was formulated that ultraviolet irradiation produced, by discrete hits, a damage localized in discrete gene-like individual "units" in each phage particle and that reactivation resulted from cooperation among the infecting particles. This cooperation was supposed to involve the same (unknown) mechanism as that involved in genetic recombination.

The requirement for reactivation in a given bacterium was then postulated to be the possession by the infecting particles as a group of at least one set of undamaged units. This led to the expression, for the maximum frequency of production of active phage,

$$y = \frac{\sum_{k=2}^{\infty} \frac{x^k e^{-x}}{k!} [1 - (1 - e^{-r/n})^k]^n}{1 - (x + 1)e^{-x}}, \quad (9-2)$$

where  $x$  = average number of inactive particles per bacterium,

$r$  = average number of hits per particle,

$k$  = an integer number, and

$n$  = number of the hypothetical units per particle (assumed in first approximation to have equal ultraviolet sensitivity).



The denominator in Eq. (9-2) is the fraction of bacteria receiving two or more particles; the numerator is the probability that the group of  $k$  particles infecting a given bacterium contains one full set of active units. It is possible to determine  $x$  and  $r$  experimentally. A comparison of  $y$  with the experimental frequency  $w$  of active phage production gave, at first, results compatible, with some limitations, with Eq. (9-2). The analysis was therefore pushed further along these lines, and estimates were given for the values of  $n$  for different phages.

The tendency of the experimental ratios  $w/y$  toward unity for small values of  $r$  (low doses) and high values of  $x$  (high multiplicities) suggested furthermore that any mechanism of recombination, if responsible for reactivation, should be an exceedingly efficient one in order to allow an essentially full utilization of needed units derived from many different phage particles in the formation of active phage. This led to the "gene-pool" hypothesis, according to which each unit reproduced independently of the others, and the resulting new units reassembled to form the new particles. This hypothesis could explain a number of features of the phage reproduction process.

Additional evidence, however, has forced revision of one basic assumption of the theory that multiplicity reactivation is due solely to a highly efficient mechanism of genetic recombination. According to theory, the minimum requirement should be the integrity of at least one full set of units in the infecting particles. If the frequency of reactivation is plotted against the dose of ultraviolet received by the particles, for high doses the curves should tend to an ultimate slope equal to the slope of the inactivation curve for the free phage since both these slopes represent the probability of persistence of one full complement of active units (Dulbecco, 1952). Analytically, it is easily seen that, for very large values of  $r$ , Eq. (9-2) tends to the form

$$y_{r \gg n} \approx \frac{\sum_{k=2}^{\infty} \frac{x^k e^{-x}}{k!} k^n e^{-r}}{1 - (x + 1)e^{-x}} = F(x)e^{-r}.$$

Dulbecco (1952), having by a special procedure obtained data on the frequency of recombination at very high radiation doses, found that for phage T2 the curves for  $w$  versus dose reach their ultimate slope much sooner than expected and that this slope is not the same as that of the inactivation curve of the single particles but only about one-fifth of it (see Fig. 9-1). This result indicates that the simple theory is inadequate.

The situation may be summarized as follows: Multiplicity reactivation represents the result of a cooperation among inactive phage particles in producing active phage. In this cooperation each particle contributes in a more than additive measure. For high ultraviolet doses, for example, bacteria with three particles have a probability of reactivation several

times greater than that of bacteria with two particles. Dulbecco's work on photoreactivation of phage (1951, unpublished results) suggests that some phages (the T2, T4, T6 group, in particular) may receive two types of ultraviolet damage, one photoreactivable by a one-quantum process, the other by a multiple-hit process. Multiplicity reactivation can overcome the effects of both types of damage. It may involve some kind of

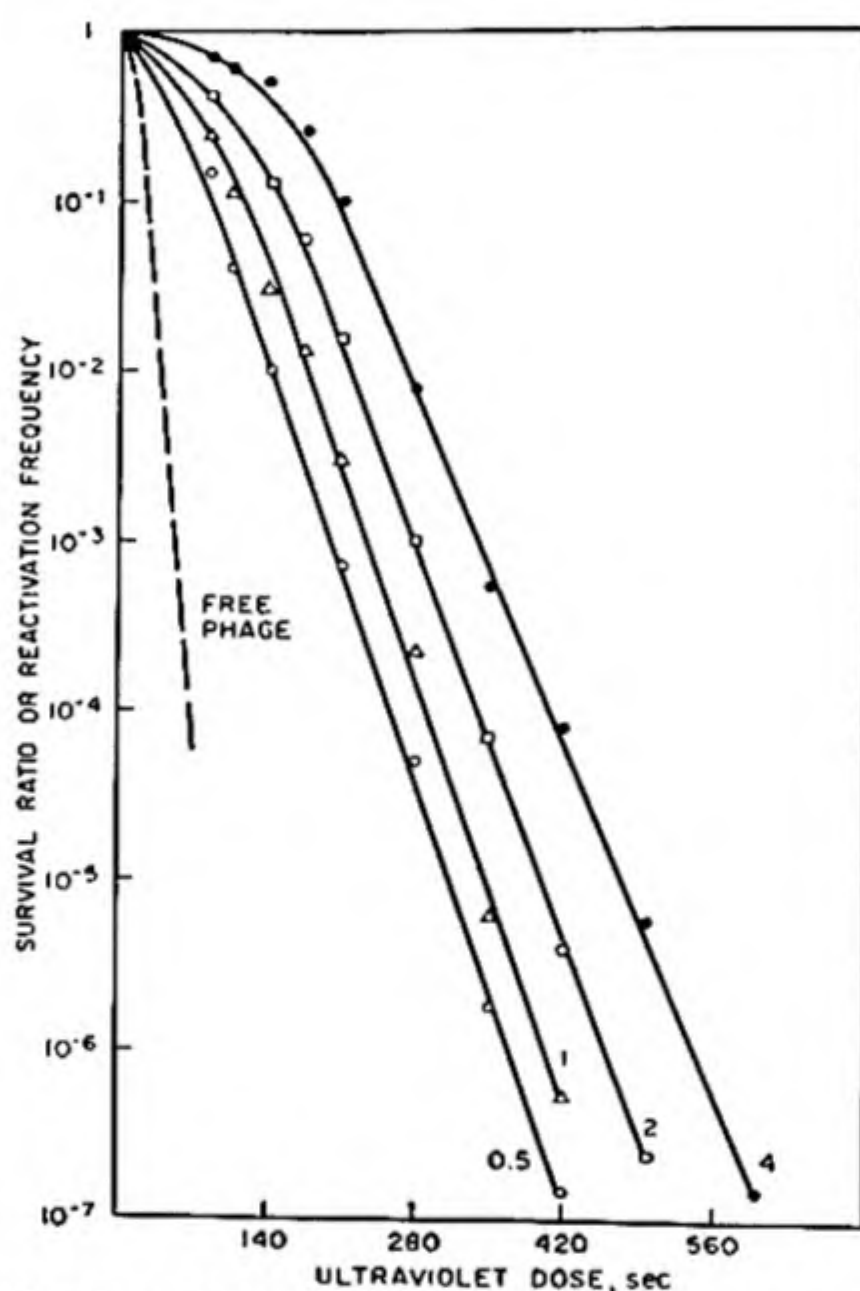


FIG. 9-1. Survival of phage T2r and its multiplicity reactivation after ultraviolet irradiation. Exposure was at 80 cm distance from a 15-watt germicidal lamp (General Electric Company). Broken line, free phage survival; solid line, reactivation frequency (fraction of multiple-infected bacteria that liberate active phage). The figures given for each solid-line curve indicate the average multiplicity of infection in the whole population. (Modified from Dulbecco, 1952.)

very efficient cooperation at the physiological level, together with a mechanism of genetic recombination of a more orthodox nature than the one postulated by the "gene-pool" theory.

For phages inactivated by X rays, multiplicity reactivation is very slight, with a frequency much lower than with ultraviolet-irradiated phage (Watson, 1950). It has been suggested that it may occur only for that fraction of particles that are inactivated by acts of X-ray adsorption which resemble ultraviolet quanta in the extent of damage they produce, and possibly in the amount of energy released. Interestingly enough, an



appreciable amount of multiplicity reactivation was found with phage inactivated by the aftereffect of X rays (see Sect. 2-1a).

## 5. INTRACELLULAR IRRADIATION OF VIRUSES

### 5-1. IRRADIATION OF CELLS INFECTED WITH EXOGENOUS VIRUSES

Irradiation of cells during infection with viruses may be of use in the study of virus reproduction. This approach has as yet been limited to bacteriophage, but it could be applied to other viruses, particularly in tissue cultures. With bacteriophage the basic experimental procedure (Anderson, 1944; Luria and Latarjet, 1947) consists in infecting a bacterial culture with virus, taking samples at intervals during the period that precedes lysis, and exposing them rapidly to various doses of radiation. The irradiated infected bacteria are then tested immediately for their ability to liberate phage. This ability can be suppressed by either ultraviolet or X rays; and, if the fraction of bacteria that liberate phage is plotted versus dose of radiation, "suppression curves" are obtained. The suppression effect is exerted on the intracellular bacteriophage rather than on the bacterial host. This is shown by the following observations:

1. Active phage can reproduce normally in bacteria exposed to ultraviolet radiation shortly before infection (Anderson, 1948).

2. If infected bacteria are irradiated immediately after infection, the rate of suppression of phage liberation as a function of radiation dose is similar to the rate of inactivation of free virus.

3. In multiple infection the suppression curve immediately after infection is of the multiple-hit type and closely resembles the curves obtained for active phage production in multiple infection with irradiated bacteriophage (see Sect. 4-2a).

As the time after infection increases, the suppression curves change in a manner characteristic for the phage. The simplest case is that of phage T7 (Benzer, 1952; see Fig. 9-2). After infection there is no change in ultraviolet sensitivity for 3 or 4 min, but then the suppression curve becomes of the multiple-hit type without any change in the final slope of the curve; this seems to indicate a simple mechanism of multiplication of virus elements having the same sensitivity as the free virus.

With phage T2 (Luria and Latarjet, 1947; Benzer, 1952) the first change in bacteria infected with one T2 particle and exposed to ultraviolet (2537 Å) is an increase in ultraviolet resistance without appreciable change in the shape of the curve. Several minutes later the inactivation curve changes to a complex type, suggesting an effect on numerous objects within each cell. In the latest stages of infection, radiation sensitivity again increases. The results suggest that phage T2 must perform an early function easily blocked by ultraviolet damage and that the radiation sensitivity increases as this early phase is passed.

Phage T2 has similarly been investigated with X rays (Latarjet, 1948). The picture is simpler than with ultraviolet since the suppression curve remains constant for several minutes, then becomes of a multiple-hit type (with lesser ultimate slope than for free phage), and finally becomes a multiple-hit curve with ultimate slope similar to that of free phage.

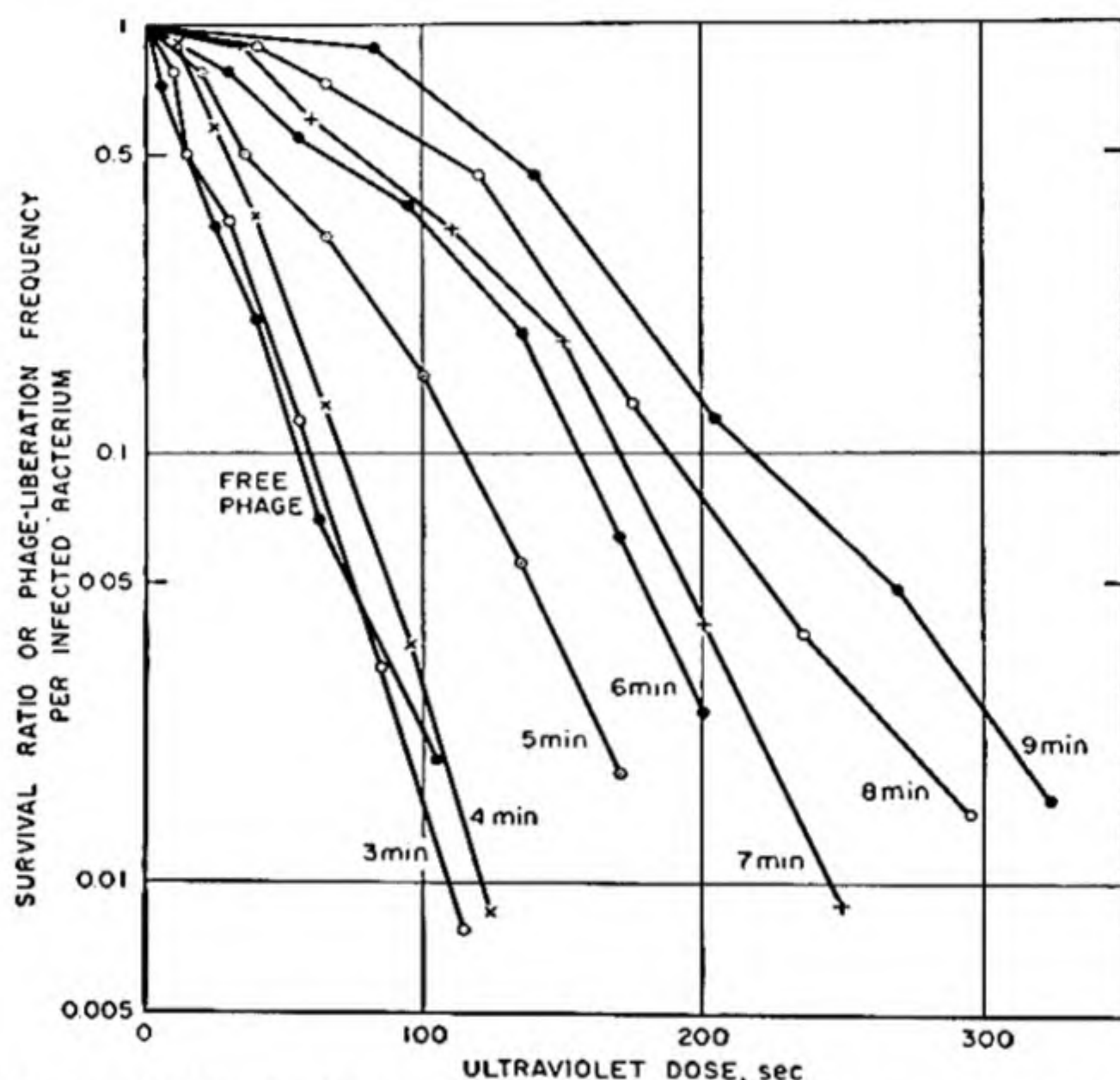


FIG. 9-2. Intracellular irradiation of phage T7. Bacteria were washed in buffer, infected with phage T7 (single infection), and then placed in a nutrient medium. Samples were taken at intervals, chilled, exposed to ultraviolet radiation, and then plated to determine the number of bacteria that still liberate phage. The suppression curves are compared with the inactivation curve for free phage. Exposure conditions were the same as for Fig. 9-1. The time given for each curve is the time between addition of nutrient and chilling previous to irradiation ("growth time"). (Benzer, 1952.)

Thus this type of analysis, although quite incomplete, suggests that a small phage such as T7 reproduces by multiplication of uniform elements, whereas T2 undergoes a complex series of changes, including a process of multiplication, which only in the latest phases leads to the presence of mature fully sensitive phage particles in the infected cells. This conclusion is in accord with all our information on the reproduction of these viruses.

The intracellular irradiation procedure allows the determination of the



stage of infection from the shape and slope of the suppression curve. This can be utilized to identify the stages at which a certain treatment stops virus reproduction. For example, on exposure of bacteria infected with phage T2 to a temperature of 45°C, the changes in ultraviolet sensitivity take place normally for the first 7 min, after which no further change occurs, as though at this time a temperature-sensitive reaction entered the picture (Benzer, 1950, unpublished data).

Although no "cure" for phage-infected bacteria has been obtained in this work, an extension of these studies to other viruses might produce results of some therapeutic value in virus infection. Even if the cells already infected could not be saved by radiation, suppression of their ability to liberate virus might prevent the spreading of infection. The value of such a procedure would depend on the relative sensitivities of the infected cells and of the normal tissues. Experiments on rabbit papilloma have shown that growth of the papillomas can be suppressed by doses of X rays much smaller than those necessary to inactivate the virus *in vitro* (Syverton, Berry, and Warren, 1941; Syverton, Harvey *et al.*, 1941). Actually, virus can be recovered in undiminished amounts from the irradiated papillomas (Friedewald and Anderson, 1943). Here the radiation probably acts on the host cell, rather than directly on the intracellular virus, in the same way as therapeutic doses of X rays affect bacterial infections by acting on the tissues of the host.

## 5-2. RADIATION AND LATENT VIRUSES

An interesting possibility is that of affecting, by means of radiation, viruses which may be present in the latent state, i.e., viruses which do not manifest themselves and which behave up to a certain point like normal cell components. The distinction between latent viruses and cell components is not always easy with the available knowledge and in some cases may actually be academic. Any self-reproducing element of the protoplasm of a cell might conceivably become a virus if by some evolutionary accident it should acquire the ability to enter other cells and there reproduce its own kind. Such an origin of viruses has been suggested, but the question will remain academic until more definite knowledge is obtained in regard to the occurrence and properties of self-reproducing units (other than the nuclear genes) in most types of cells. Should such units be more widespread than they appear to be, their origin in the process of cell evolution would still be unknown. It is known that a virus may enter a cell and reproduce while the cell goes through several cell generations, often without causing recognizable cell disturbances. Such a virus behaves at least for some time as a cell component. This type of symbiosis often prolongs itself for many cell generations, and in multicellular organisms some viruses are transmitted through the gametes from generation to generation. The recognition of the virus depends then only on indirect

tests, either the inoculation of tissue extracts in a virus-susceptible host or the search for antiviral antibody in the latently infected organisms. The distinction between virus and cell component is thus quite difficult. The information relevant to this area of biology cannot be discussed here. In connection with the possibility of affecting latent viruses or self-reproducing cell components within cells as a means of analyzing the relation between these entities and the cell as a whole, some radiation results are pertinent.

5-2a. *Irradiation of Lysogenic Bacteria.* Lysogenic bacteria carry one or more bacteriophages in a latent form without recognizable manifestations. In these bacteria the phage is apparently present as immature virus or "prophage." Occasionally a lysogenic cell is lysed and liberates a cluster of mature phage particles, whose presence can be recognized if a susceptible strain of bacteria that responds to phage infection by lysis is available (Lwoff and Gutmann, 1950). In the course of attempts to define the conditions that lead to the occasional maturation of virus in the lysogenic bacteria, it has been discovered (Lwoff *et al.*, 1950) that ultraviolet irradiation produces in some lysogenic strains a massive lysis accompanied by liberation of mature phage. This suggests that something in the lysogenic bacteria prevents the maturation of prophage into bacteriophage, thus preserving the symbiotic relation, and that ultraviolet, by removing the inhibition, releases the maturation process.

5-2b. *Irradiation and Cytoplasmic Factors.* Another pertinent observation concerns the destruction by radiation of the cytoplasmic factor "kappa" in *Paramecium aurelia*. Some strains of this organism produce a poison (paramecin) which is lethal for individuals of other strains. The production of the poison is always associated with the presence in the cytoplasm of the killer animals of peculiar Feulgen-positive particles, 0.3–0.8  $\mu$  in size, which are the material carriers of a genetically recognizable self-reproducing mutable factor (kappa). The continuous production of kappa depends both on the presence of preexistent kappa and on the proper genetic background. Kappa has been transmitted from one individual to another by "infection" with cell extracts, thus resembling a virus or a rickettsia (Sonneborn, 1949).

X rays (Preer, 1950), as well as ultraviolet (Sonneborn, personal communication) and nitrogen mustards (Geckler, 1949), eliminate the killer factor from the protoplasm at a rate that suggests a one-hit inactivation process with an inactivation dose of approximately 4000 r. This dose is comparable to the doses required for sterilization of bacteria which are somewhat larger in size than the kappa particles. This result suggests interesting applications of radiation analysis to the study of cytoplasmic inheritance and encourages speculation on the possible use of selective effects of radiation on cytoplasmic elements in modifying development and differentiation (which have been suggested to be controlled by cyto-



plasmic determinants of heredity) and in altering the neoplastic properties of tumors.

It may be of interest to mention that another self-reproducing mutable cytoplasmic factor, the virus-like "genoïde" for carbon dioxide sensitivity in *Drosophila* (L'Héritier, 1949), which can be transmitted from fly to fly by cell-free extracts, is inactivated in the extracts by X rays. The inactivation dose is around  $10^5$  r, similar to that for medium-sized viruses (L'Héritier and Plus, 1950).

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## CHAPTER 10

### Effects of Radiation on Bacteria

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*Bactericidal effects of radiation. High-energy radiations: General results of quantitative investigations. Factors influencing sensitivity to ionizing radiations: Ultraviolet radiation—Extreme ultraviolet radiation—Near-ultraviolet and short-visible radiation. Physiological properties of bacteria following irradiation. Sublethal effects of radiation. Bacterial genetics: Radiation-induced mutations in bacteria—Mechanism of radiation effects.*

One of the first laboratory observations of the effects of radiations on bacteria was published by Downes and Blunt in 1877. Since that date a voluminous literature has accumulated as a result of the wide interest in the effects of radiation on bacteria *per se* and also because of the relative ease with which quantitative studies of the biological effects of radiation can be made with bacteria.

Despite the numerous investigations during the past seventy-five years, understanding of the effects of radiation on bacteria is still only fragmentary. Furthermore, the advances since the 1936 predecessors of these volumes, especially during the past decade, have been rapid in comparison to the advances in earlier work. For example, almost all knowledge of radiation-induced mutations in bacteria has been gained since that time. Consequently, in the limited space available, no attempt will be made to review completely the earlier literature. Rather, only those earlier contributions considered to be especially significant will be included with correspondingly greater emphasis on recent research. The rather numerous reviews concerned with various aspects of the biological effects of radiation combine to give a complete coverage of the development of the field

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(Duggar, 1936; Giese, 1945, 1947, 1950; Ellis *et al.*, 1941; Latarjet, 1946; Lea, 1947; Loofbourow, 1948; Mitchell, 1951; Rahn, 1945).

### BACTERICIDAL EFFECTS OF RADIATION

When bacteria are exposed to radiation, in either the high-energy or the ultraviolet range, the most prominent effect is the apparent killing of a percentage of the cells, the fraction killed being a function of the absorbed energy. The usual criterion of survival is the ability of the bacteria to form a colony visible to the eye when incubated following plating on ordinary culture media. This arbitrarily adopted measure of the bactericidal effects of radiation, although convenient for quantitative studies, is influenced by a variety of experimental conditions. Hollaender (1943) showed that prolonged exposure to saline, following long-ultraviolet or short-visible irradiation, reduced the fraction of colony-forming organisms. Roberts and Aldous (1949) made careful studies of various experimental conditions both before and after ultraviolet irradiation which affect the survival of *Escherichia coli*, strain B. Their results will be considered in more detail later, but for this strain, a hundredfold variation in survival could be produced by changing the postirradiation treatment. Furthermore, the shape and slope of the survival curve were markedly influenced by different conditions. Strain B/r, a radiation-resistant mutant derived from strain B (Witkin, 1946, 1947), did not show similar variation in survival when subjected to the same experimental treatments. Kelner (1949a, b) observed that exposing bacteria to visible light following exposure to ultraviolet significantly increased survival (photoreactivation). Anderson (1949, 1951b) and Stein and Meutzner (1950) have shown that increasing the temperature of incubation increases the survival of ultraviolet-irradiated *E. coli* B. Survival following X irradiation was shown by Hollaender, Stapleton, and Martin (1951) to be influenced by the oxygen concentration of the medium at the time of irradiation. Stapleton *et al.* (1953) have discovered that incubation at suboptimal temperatures markedly increases the survival of bacteria exposed to X rays, and Stapleton (1952) has found marked differences in the radiosensitivity of *E. coli* cells at different stages of the growth cycle.

These findings are mentioned to emphasize the multiplicity of factors which influence the quantitative results obtained in studies of the bactericidal effects of radiation. Consequently, in order to obtain reproducible results, it is necessary that these variables be adequately controlled. Furthermore, it is impossible to determine how much of the variation in the results obtained by different investigators is attributable to differences in their experimental techniques. This is especially true in regard to many of the earlier studies.

Bactericidal effect, lethal effect, killing, and inactivation are used

synonymously to indicate the failure of the cells to form a colony visible to the naked eye when plated under the particular conditions of the experiment under discussion.

## HIGH-ENERGY RADIATIONS

### GENERAL RESULTS OF QUANTITATIVE INVESTIGATIONS

Minch in 1896 was apparently the first to attempt to study the bactericidal effects of X rays. His results were essentially negative as were those of many other investigators during the next thirty years. Low intensities of X rays and rather insensitive bacteriological techniques seem to be the chief reasons for the conflicting and often negative results of different investigators during this period. Duggar (1936) briefly discusses these early X-ray results, and the bibliographies given by him and by Pugsley *et al.* (1935) form a helpful guide to the early literature.

During this period, however, it was convincingly demonstrated by a number of investigators that ionizing radiations do exert a marked bactericidal effect. Green (1904), employing semiquantitative bacteriological techniques, studied the bactericidal effects of radium  $\beta$  rays on 23 species of bacteria including five spore formers. In his experiments all species were killed by the  $\beta$  rays, and the spore formers were found to be considerably more resistant than the vegetative forms. One of the outstanding contributions during this period was that of Chambers and Russ (1912). These workers studied the effects of radium emanation, primarily  $\beta$  rays, on distilled water suspensions of *Staphylococcus aureus*, *Escherichia coli*, *Bacillus pyocyaneus*, and *Bacillus anthracis*. A pronounced bactericidal action was observed with all species; anthrax spores were observed to be the most resistant to radiation. Quantitative estimates of surviving organisms made by plate counts of an irradiated *S. aureus* suspension, when plotted semilogarithmically, gave rise to a straight line. This is the first exponential survival curve reported for bacteria subjected to radiation. Furthermore, these workers observed motile cells of *B. pyocyaneus* in irradiated suspensions in which no colony-forming organisms were present. Similar observations of inactivated but motile cells have been made by Bruynoghe and Mund (1925).

Following application of the target theory by Crowther (1924, 1926) to inhibition of mitosis in tissue culture cells observed by Strangeways and Oakley (1923) and to his own data on killing of *Colpidium colpoda*, a number of investigators applied similar analyses to the bactericidal effects of ionizing radiations. Holweck (1929) and Lacassagne (1929) irradiated "pyocyanique S" with soft X rays of 4 and 8.3 Å wave lengths. They observed exponential killing with 4 Å X rays, but a multihit or sigmoidal type of survival curve was obtained with 8.3 Å. However, Lea, Haines, and Coulson (unpublished, see Lea, 1947) observed exponential sur-



vival curves when repeating this work with the same strain and wave lengths.

Wyckoff (1930a, b) and Wyckoff and Rivers (1930), in a series of careful studies, more firmly established the occurrence of exponential survival curves following exposure to ionizing radiations. Wyckoff and Rivers (1930) studied the bactericidal effects of 155-kv  $\beta$  rays on *E. coli*, *Salmonella typhimurium*, and *Staphylococcus aureus* cells seeded on the surface of agar plates. Exponential survival curves were obtained for all except *S. aureus*. By allowing *E. coli* to divide before irradiation, they showed that clumping of the cells was probably the reason that exponential survival curves were not obtained for *S. aureus*. They conclude that a single electron is sufficient to inactivate a cell of these species. Similar results (Wyckoff, 1930a) were obtained in studies with *E. coli* and *S. typhimurium* using copper-K X rays and the soft general radiation from a tungsten tube operated at 12 kv. Later, Wyckoff (1930b) studied the killing of *E. coli* with X rays of wave lengths varying from 0.5 to 4 Å. Exponential killing was observed at all wave lengths. Wyckoff interpreted his results to indicate that a single quantum of X rays was sufficient to kill the bacteria. His estimated values of the sensitive volume of the organisms decreased with increasing wave lengths as a result of the greater incident energy required for inactivation at the longer wave lengths.

Similar results have been obtained by other workers with various bacterial species subjected to various ionizing radiations. Hercik (1933, 1934b) observed exponential survival curves with *Serratia marcescens* irradiated with  $\alpha$  particles emitted by polonium. Pugsley *et al.* (1935) observed exponential killing for *E. coli* irradiated with 40-kvp X rays but obtained sigmoidal curves for *Sarcina lutea*. A correction applied for the degree of clumping as determined by microscopic examination of the irradiated suspension resulted in an exponential survival curve. Lorenz and Henshaw (1941) made extensive tests of the bactericidal effects of 200-kvp X rays on *Achromobacter fischeri*. Statistical analysis showed no systematic deviation from an exponential survival curve. During the past decade, in which there has been almost a routine use of radiation for the induction of mutations in microorganisms, numerous investigators have observed exponential survival curves (e.g., Lincoln and Gowen, 1942; Demerec and Latarjet, 1946; Witkin, 1947; Roepke and Mercer, 1947; Anderson, 1951a). Fram *et al.* (1950) report exponential survival curves for six species following irradiation with 50-kvp X rays. A typical exponential survival curve with 5 per cent confidence limits of the plotted points is shown in Fig. 10-1 (Stapleton, unpublished data).

Not all investigators have observed exponential survival curves, however. Observation by Holweck (1929) and Lacassagne (1929) of sigmoidal survival curves with 8.3 Å X rays has already been mentioned. Claus (1933) observed sigmoidal survival curves for *E. coli* following

X irradiation in the presence of heavy metal ions. The sigmoidal curves may have been due to the short wave lengths of the secondary radiations which were considered to be the main cause of inactivation. Luria (1939), employing the same bacteriological techniques in both cases, observed exponential survival curves with polonium  $\alpha$  particles and a two-hit sigmoidal curve with 0.7 A X rays. Microscopic examination of the irradiated cells of *E. coli* revealed that, although some of the cells were killed immediately and did not divide or grow, others continued to grow without dividing and ultimately developed into long filamentous forms. These filamentous forms either divided a few times and then died or else recovered and proceeded to develop normal colonies. Exposure to both  $\alpha$  and X radiation caused filamentous forms, but the proportion was higher with X rays. Luria points out that death by several mechanisms is incompatible with the simple mathematical formulation of the target theory.

Similar results with *Aspergillus terreus* spores have been reported by Stapleton, Hollaender, and Martin (1952) and Zirkle *et al.* (1952) who report sigmoidal survival curves with hard X rays and exponential curves with densely ionizing protons and  $\alpha$  particles. Since in both cases air-dried spores were irradiated, there is no possibility that production of a toxic substance in the irradiated suspending medium could be responsible for the sigmoidal curves.

Stapleton (1952) made the important observation that the form of the survival curve obtained after X irradiation (250 kvp) of *E. coli* B/r cells depends on the stage of the growth cycle of the culture. Cells from fully grown cultures in the stationary phase yield exponential survival curves (Fig. 10-1). However, when cells in the lag phase were exposed to X rays, sigmoidal survival curves were obtained, the deviation from exponential killing increasing to a maximum at the end of the lag phase. Interpretation of the sigmoidal curves on the multitarget theory (Atwood and Norman, 1949) shows an increase from one to about eight targets during the

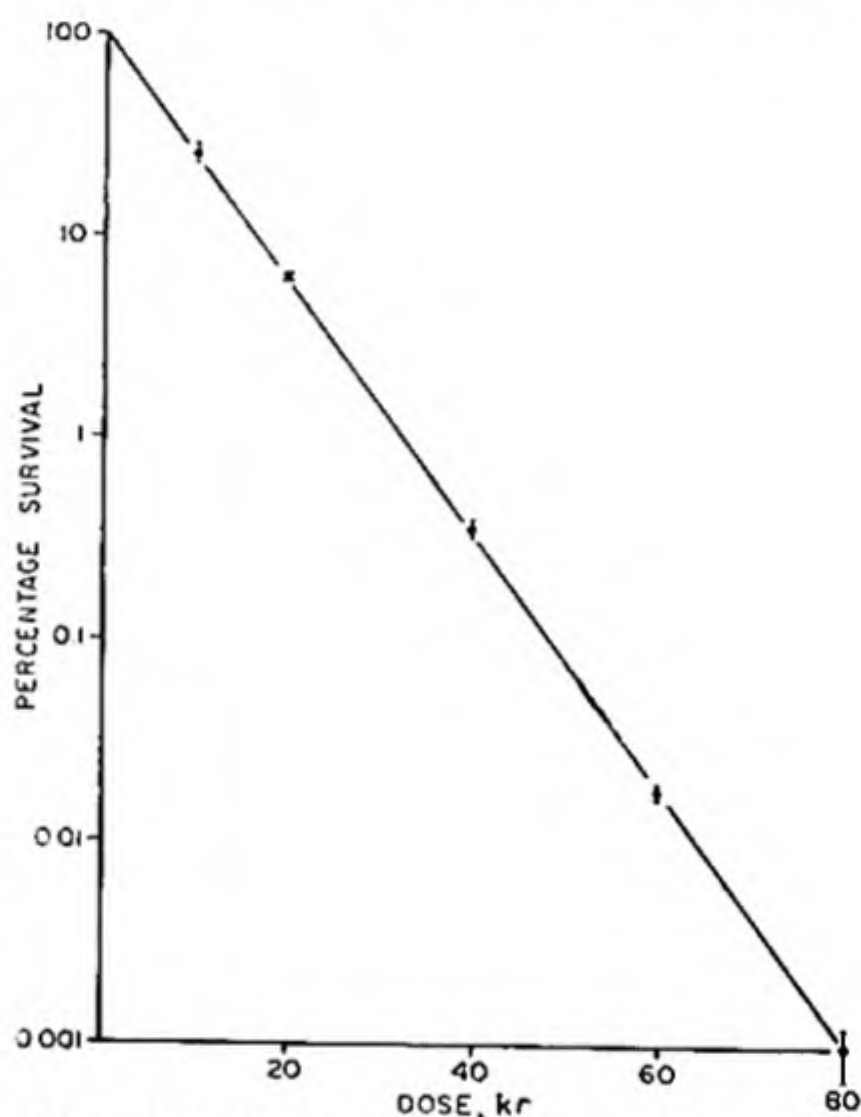


FIG. 10-1. Exponential survival curve with 5 per cent confidence limits for each point; *E. coli* B/r resting cells irradiated in air-saturated buffer with 250-kvp X rays. (Stapleton, unpublished data.)



lag phase. Cytological studies show a high correspondence between the number of observable nuclear bodies and the number of targets estimated for cells in different growth phases. The survival curves become exponential and the target number becomes 1 as growth proceeds through the logarithmic phase and the stationary phase is approached. Stapleton made the further interesting observation that stationary-phase cells subjected to  $\gamma$  rays from a  $\text{Co}^{60}$  source exhibit a "two-hit" killing curve as compared to an exponential curve for X rays.

Lea *et al.* (1936), in addition to discussing the principal theories of the mechanism of the bactericidal action of ionizing radiations, present important quantitative data on survival curves of irradiated bacteria. They studied primarily the survival of *Bacillus mesentericus* spores and, less extensively, the survival of *E. coli* and *S. aureus*. The radiations employed were  $\alpha$  particles emitted by polonium and  $\beta$  rays produced by radon disintegration. Careful energy measurements were made and the geometrical conditions were controlled. The spores and bacteria were irradiated in dried gelatin films of approximately 1- $\mu$  thickness. The fraction of organisms surviving was found to be a diminishing exponential function of the time of exposure, or dose, for all organisms with both radiations. The target areas and mean lethal doses (MLD) were computed for each organism for both  $\alpha$  and  $\beta$  rays.

The studies with *B. mesentericus* spores and *E. coli* were extended to include radium  $\gamma$  rays (Lea *et al.*, 1937), neutrons, hard X rays of an effective wave length of 0.15 A, and soft X rays of 1.5, 4.1, and 8.3 A (Lea *et al.*, 1941). The organisms were irradiated in aqueous suspensions; suspending the cells in previously irradiated distilled water given comparable exposures was shown to have no effect. Again, exponential survival curves were observed in all cases.

Spear (1944), utilizing the same bacterial strains employed by Lea and his associates, presents the most extensive data on the bactericidal effects of neutrons. The neutron source was a beryllium target bombarded by 8-Mev deuterons accelerated in a 37-in. cyclotron. Aqueous suspensions were exposed, and the dose was measured with a Victoreen dosimeter calibrated in roentgens. The survival curve did not depart systematically from an exponential curve. The ratio of the  $\gamma$ -ray dose in roentgen units to the neutron dose in n units required to produce 50 per cent lethality was 3.2 for *E. coli* and 5.3 for *B. mesentericus* spores. Because of the lack of absolute dosimetry with neutrons, Spear presents a curve (Fig. 10-2) showing a systematic decrease in the MLD ratio of *B. mesentericus* spores to *E. coli* cells as the ionization density increases. This curve includes all the data of Lea and his associates and shows that the neutron data fit into the general relation.

No effect of varying temperature between 2.5° and 36°C at the time of irradiation was observed by Hercik (1934a) in studies employing  $\alpha$  par-

ticles and *Serratia marcescens*. Lea *et al.* (1936) found the rate of inactivation of *B. mesentericus* spores by  $\alpha$  and  $\beta$  particles to be independent of temperature between  $-20^{\circ}$  and  $+50^{\circ}\text{C}$ . No influence of temperature was noted on the rate of inactivation of *S. aureus*. These results differ markedly from the high temperature coefficients experienced with chemical disinfectants. Lea *et al.* (1937) showed that varying the temperature from  $0^{\circ}$ – $37^{\circ}\text{C}$  had no influence on the survival of *E. coli* exposed to  $\gamma$  rays.

Lea *et al.* (1936) observed that a sixfold variation in intensity of  $\alpha$ -particle flux had no effect on the proportion of organisms surviving a given dose. This was confirmed by experiments in which a given dose was administered in a number of fractions, no effect on survival being observed.

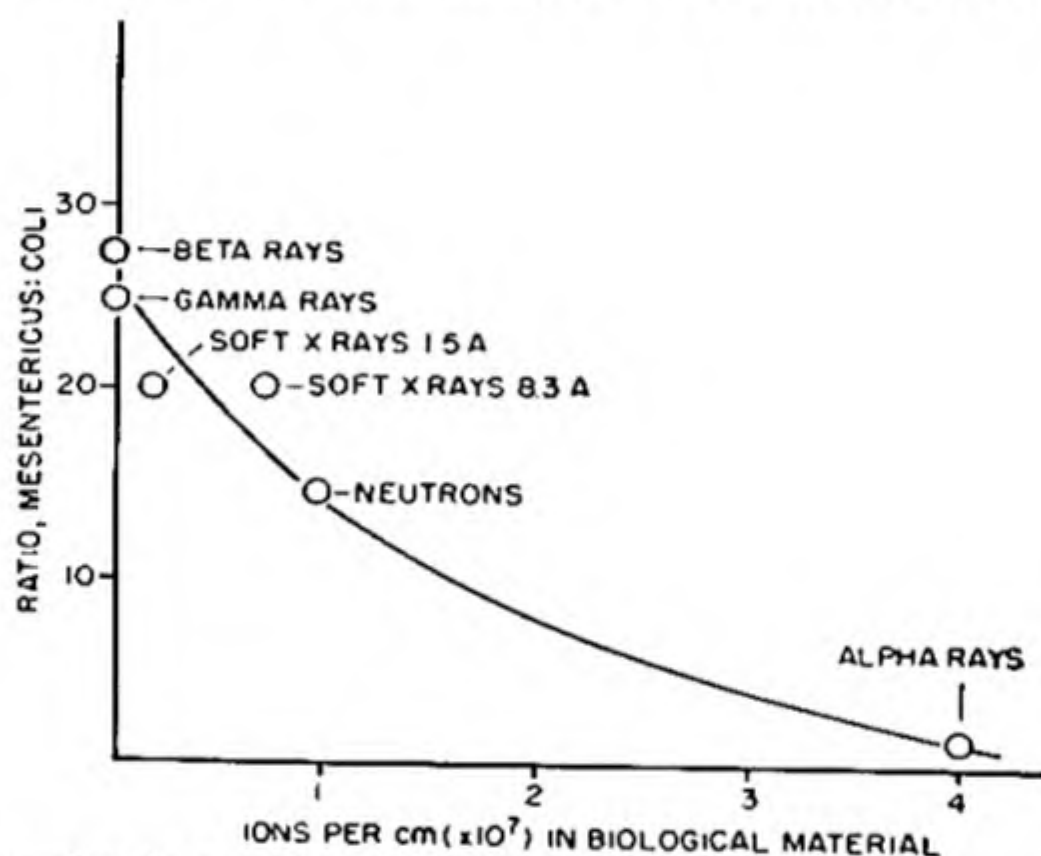


FIG. 10-2. Curve showing ratio of MLD mesentericus:MLD coli plotted against ion density for a number of different radiations. (Adapted from Spear, 1944.)

Lea *et al.* (1941) reported further data on the lack of an intensity effect of  $\alpha$  particles and 8.3 A X rays. A seventy-fivefold variation in intensity was employed in the latter case. Extremely high intensities of  $\beta$  particles have been shown by Huber (1951) to be bactericidally effective.

The observation of filamentous forms in bacterial cultures following ultraviolet irradiation was reported by Gates (1933) who concluded that the mechanism of cell division was more sensitive to radiation than the processes of growth. Similar observations have been reported by several investigators (Spencer, 1935; Luria, 1939; Witkin, 1947; Roberts and Aldous, 1949). Lea *et al.* (1937) observed such filamentous forms following continuous  $\gamma$  irradiation of growing *E. coli* cultures. Subsequent experiments indicated that the filamentous cells reacted to  $\gamma$  rays in the same manner as the normal cells, i.e., they were equally sensitive to radiation and were inactivated in an exponential manner.

Careful studies of the effect of ionization density on the biological effect-



tiveness of polonium  $\alpha$  particles have been reported by Zirkle (1940) who obtained different ionization densities by utilizing different portions of the path length. An increase in effectiveness with increasing ionization density was observed with *Aspergillus niger* spores whereas, for inactivation of *E. coli*, an inverse relation was observed. Little influence of ionization density on survival of yeast cells was noted.

In Table 10-1 are assembled the available data on the relation of ionization density and the bactericidal effect of radiations (see also Fano, Chap. 1, and Zirkle, Chap. 6, volume I of this series). The estimates made from Spear (1944) and Zirkle (1940) are only approximate but serve to indicate the general relations. It will be observed that the MLD (37 per cent survival dose) for *E. coli* increases as the ionization density of the radiation increases. On the contrary, the MLD for *B. mesentericus* and for *A. niger* spores decreases with increasing ionization density. This difference is as yet unexplained, and it may indicate that different mechanisms are involved in the lethal effects of irradiation for the two-cell forms. The increased efficiency of more densely ionizing radiations in killing *A. terreus* spores is confirmed by Stapleton, Hollaender, and Martin (1952) who add the further interesting observation that more densely ionizing  $\alpha$  particles are less efficient than hard X rays in inducing mutations.

Summarizing, three general conclusions seem warranted: (1) Both exponential and sigmoidal survival curves may be observed, depending on the bacterial strain, the technique of irradiation, the characteristics of the radiation, and the stage of growth of the irradiated cells. (2) The surviving fraction for a given dose of radiation is independent, within limits, of the intensity of the incident radiation or of the fractionation of the dose. (3) For vegetative cells the bactericidal effectiveness of a given dose decreases with increasing ionization density; the opposite seems to be true for spores.

The interpretation to be placed on these general results is not clear at this time. Lea *et al.* (1936) and Lea (1947) have discussed at length the various interpretations proposed for the observed survival curves. Interpretation in accordance with the target theory seems the most plausible since the first-order kinetics are a natural consequence of the theory. Similarly, the sigmoidal survival curves are also easily accounted for on the basis of either multiple hits required in one target or single hits in multiple targets. Atwood and Norman (1949) discuss this latter interpretation. Lea (1947) especially has been a strong proponent of the target theory. He interprets the results to indicate that a single ionization is sufficient to inactivate a bacterial cell. He has developed the hypothesis, first suggested by Rahn (1929, 1930), that the bactericidal effects are due to lethal mutations induced by the radiation. Following elaborate analysis of the results obtained with *E. coli* exposed to radiations

TABLE 10-1. MEAN LETHAL DOSES<sup>a</sup> OF VARIOUS RADIATIONS ON VARIOUS BACTERIA

Reference	Radiation	MLD
<i>E. coli</i> (doses in 10 <sup>3</sup> r or 10 <sup>3</sup> n)		
Wyckoff (1930b).....	X rays, A:	
	0.56	4.2
	0.71	4.6
	1.5	4.3
	2.3	6.7
	4.0	8.4
Zirkle (1940).....	X rays 0.3 A	3.9
	$\alpha$ Particles, Mev:	
	~5	5.7
Lea <i>et al.</i> (1941).....	~2	6.4
	$\beta$ rays	4
	$\gamma$ rays	5.2
	X rays, A:	
	0.15	6.0
	1.5	6.5
	8.3	7.5
	Neutrons	7.1 (4.1) <sup>b</sup>
	$\alpha$ Particles	24
<i>B. mesentericus</i> spores (doses in 10 <sup>5</sup> r)		
Lea <i>et al.</i> (1941).....	$\beta$ rays	1.1
	$\gamma$ rays	1.3
	X rays, A:	
	1.5	1.3
	4.1	1.1
	8.3	1.5
	Neutrons	0.61 <sup>b</sup>
	$\alpha$ Particles	0.26
<i>A. niger</i> spores <sup>c</sup> (doses in 10 <sup>4</sup> r)		
Zirkle (1940).....	$\alpha$ Particles, Mev:	
	~4	11.0
	~3	3.6
	~2	4.7
	~1.5	3.9
	~1	3.2

<sup>a</sup> MLD, 37 per cent survival dose.<sup>b</sup> Estimated from Spear (1944).<sup>c</sup> Spore germination, not colony growth, was measured; estimated from Zirkle (1940).



of different ionization densities, Lea (1947) concludes that the bactericidal effects can be accounted for on the hypothesis of lethal mutations induced among 250 genes having an average diameter of 12 m $\mu$ .

An increasing amount of evidence has been accumulating to indicate that a major portion of the effects of ionizing radiations may be indirect. Consequently there is considerable doubt that the target theory, without modification, may be validly applied to interpretation of those bactericidal phenomena in which indirect actions are known to be involved. Further discussion is given in a later section.

### FACTORS INFLUENCING SENSITIVITY TO IONIZING RADIATIONS

Few comparisons have been made of the X-ray sensitivity of spores and the parent vegetative forms. In general, spores have been found to be more resistant (Green, 1904; Chambers and Russ, 1912; Baker, 1935; Lea *et al.*, 1936, 1941). The greater resistance to radiation of spores may be partially due to the lower water content (Stapleton and Hollaender, 1952). Of equal interest is the generality of the inverse relation of sensitivity of spores and vegetative cells to radiations of different ionization densities (Table 10-1).

The relative sensitivity of *E. coli* B/r cells to 250-kvp X rays at different stages of the growth cycle has been studied by Stapleton (1952), as discussed earlier. There is a sharp decrease in sensitivity during the lag phase, followed by a marked increase in sensitivity during the logarithmic phase. The maximum sensitivity is reached at the end of the logarithmic phase, and as the stationary phase progresses, the sensitivity gradually declines to the initial level.

TABLE 10-2. MEAN LETHAL DOSES<sup>a</sup> OF *Escherichia coli* STRAINS IRRADIATED DURING THE STATIONARY PHASE IN AIR (INCUBATED AT 37°C AND IRRADIATED WITH 250-KVP X RAYS)  
(From Stapleton, personal communication.)

Strain	MLD, (r) $\times 10^3$
B.....	3.5
B/r.....	6.2
Tennessee.....	6.0
86G.....	6.2
Gratia.....	6.5
H-52.....	6.5
Crook.....	8.0
Texas.....	10.0

<sup>a</sup> MLD, 37 per cent survival.

The relation of genetic constitution to radiation resistance will be discussed later. That strains within a species may vary widely in sensitivity to radiation is shown in Table 10-2, in which the MLD values for eight *E. coli* strains exposed to 250-kvp X rays are shown (Stapleton, personal

communication). Although strain B/r shows about the median resistance in this group, strain B is significantly more sensitive than any of the others.

*Relation of Oxygen Concentration to X-ray Effects.* The current interest in the relation of oxygen tension to the effects of X rays on living cells stems largely from the work of Thoday and Read (1947), although earlier investigators had made similar observations. The relation of oxygen concentration to cytogenetic effects is discussed by Giles (Chap. 10, volume I of this series) along with possible interpretations to be placed upon the observations.

A similar relation between oxygen concentration and bactericidal effectiveness of X rays has been observed for *E. coli* B/r by Hollaender and coworkers (Hollaender, Stapleton, and Martin, 1951; Hollaender, Baker, and Anderson, 1951; Hollaender, Stapleton, and Burnett, 1951; Hollaender and Stapleton, 1953). This same group has obtained interesting results on the closely allied problem of chemical protection against X rays.

Except when studying temperature effects, all irradiations were performed at 2°C with washed cells suspended in *M*/15 phosphate buffer. Reduction of oxygen tension was accomplished by partial evacuation followed by saturation with nitrogen, helium, hydrogen, or carbon dioxide. The particular gas used to replace oxygen was of no importance.

Figures 10-3 and 4 from Hollaender, Stapleton, and Martin (1951) show that lowering the oxygen tension changes not only the slope of the survival curve but also the shape, although in a later publication (Burnett *et al.*, 1951) exponential survival curves are shown for both oxygen-saturated and nitrogen-saturated suspensions as well as for suspensions containing 0.04 *M* concentrations of sodium hydrosulfite ( $\text{Na}_2\text{S}_2\text{O}_4$ ), British anti-Lewisite (BAL), and ethanol. Equally apparent in Figs. 10-3 and 4 is the lower sensitivity of cells grown in glucose broth and of cells grown anaerobically, in contrast to the sensitivity of aerobically grown cells. Although the ultimate slope of the nitrogen-saturation survival curves is less steep than the slope of those for oxygen saturation, cells grown in glucose broth or nutrient broth, either aerobically or anaerobically, exhibit parallel survival curves once the threshold dosage is exceeded. The threshold dose is a function of the method of culturing.

Hollaender, Stapleton, and Martin (1951) observed that cells irradiated in oxygen-saturated suspensions were more sensitive at 2°C, whereas cells irradiated in the absence of oxygen were more sensitive at 37°C. As discussed earlier, Hercik (1934a) and Lea *et al.* (1936) failed to detect an effect of temperature. The apparent disagreement is probably attributable to the solubility of oxygen at different temperatures and to differences in the technique of irradiation, since the latter workers exposed the cells either on the surface of dried agar plates or in dried films of gelatin. Hollaender, Stapleton, and Martin (1951) further observed that the cells



were more sensitive as their concentration in the irradiated suspensions was reduced and that nutrient broth and certain amino acids afforded some protection. All these observations indicate that an indirect action of X rays is involved in bacterial inactivation, which may be similar to the indirect action of X rays on dilute suspensions of enzymes and viruses (for discussion, see Lea, 1947).

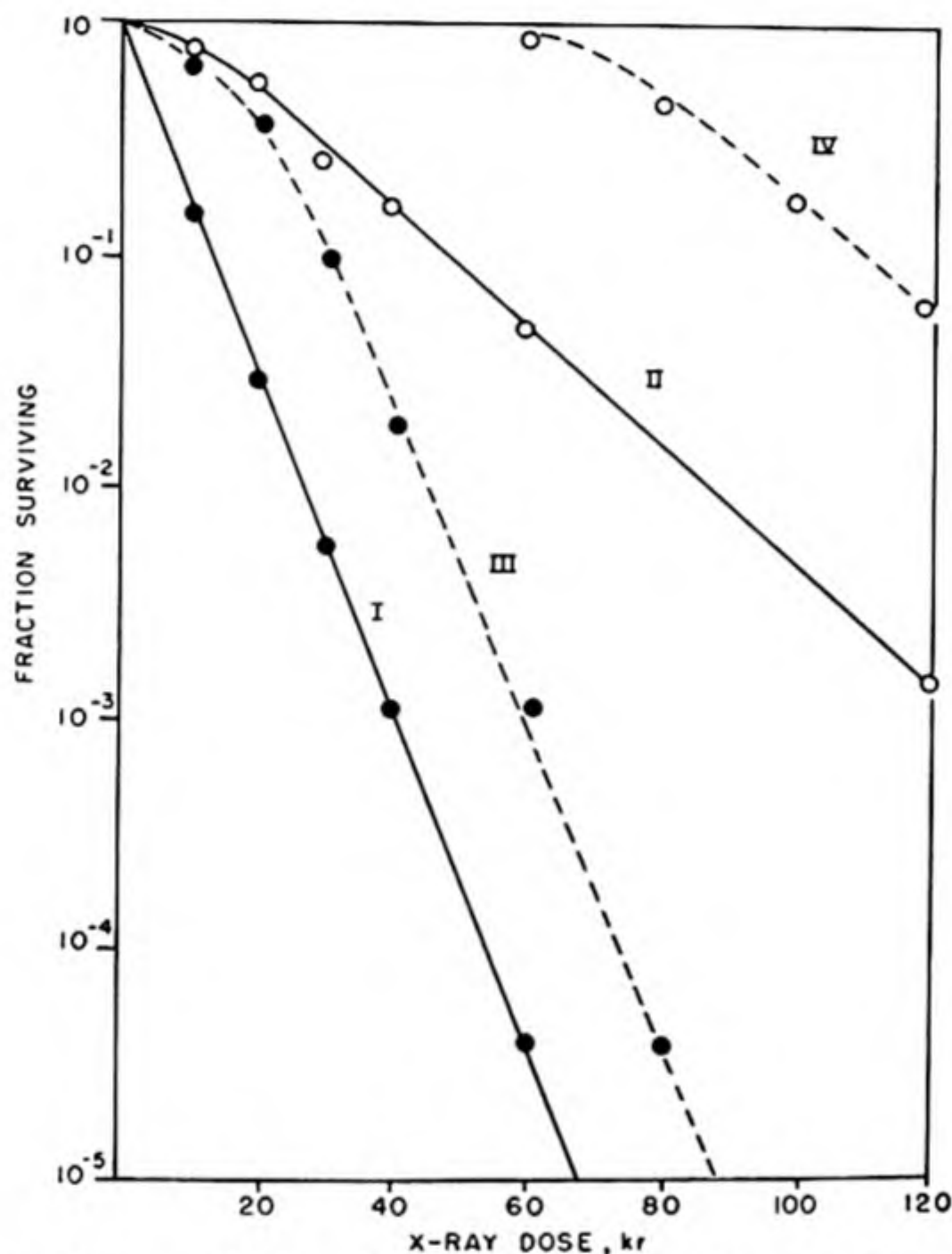


FIG. 10-3. Comparative sensitivity of aerobic and anaerobic cells irradiated in high and low oxygen tensions. I, aerobic broth cells irradiated in oxygen-saturated buffer; II, aerobic broth cells irradiated in nitrogen-saturated buffer; III, anaerobic glucose cells irradiated in oxygen-saturated buffer; IV, anaerobic glucose cells irradiated in nitrogen-saturated buffer. (Hollaender, Stapleton, and Martin, 1951.)

Current experiments (Stapleton, personal communication) indicate that since the cell suspensions in the earlier studies were prepared at room temperature and were not chilled until just before irradiation, the apparent decrease in sensitivity with increased bacterial concentration may be attributed to the greater decrease in oxygen concentration resulting from endogenous oxygen utilization by the cells in the more concentrated suspensions. The existence of a critical or threshold concentration of oxygen below which survival is greatly increased is indicated by Fig. 10-5 (from Morse, Burke, and Burnett—see Hollaender and Stapleton,

1953) which nicely shows the relation between dissolved oxygen at the time of irradiation and survival of X-irradiated *E. coli* B/r cells.

*Chemical Protection against X rays.* Some of the more salient findings in the studies on chemical protection against X rays are illustrated in Table 10-3, adapted from Hollaender and Stapleton (1953), and in the

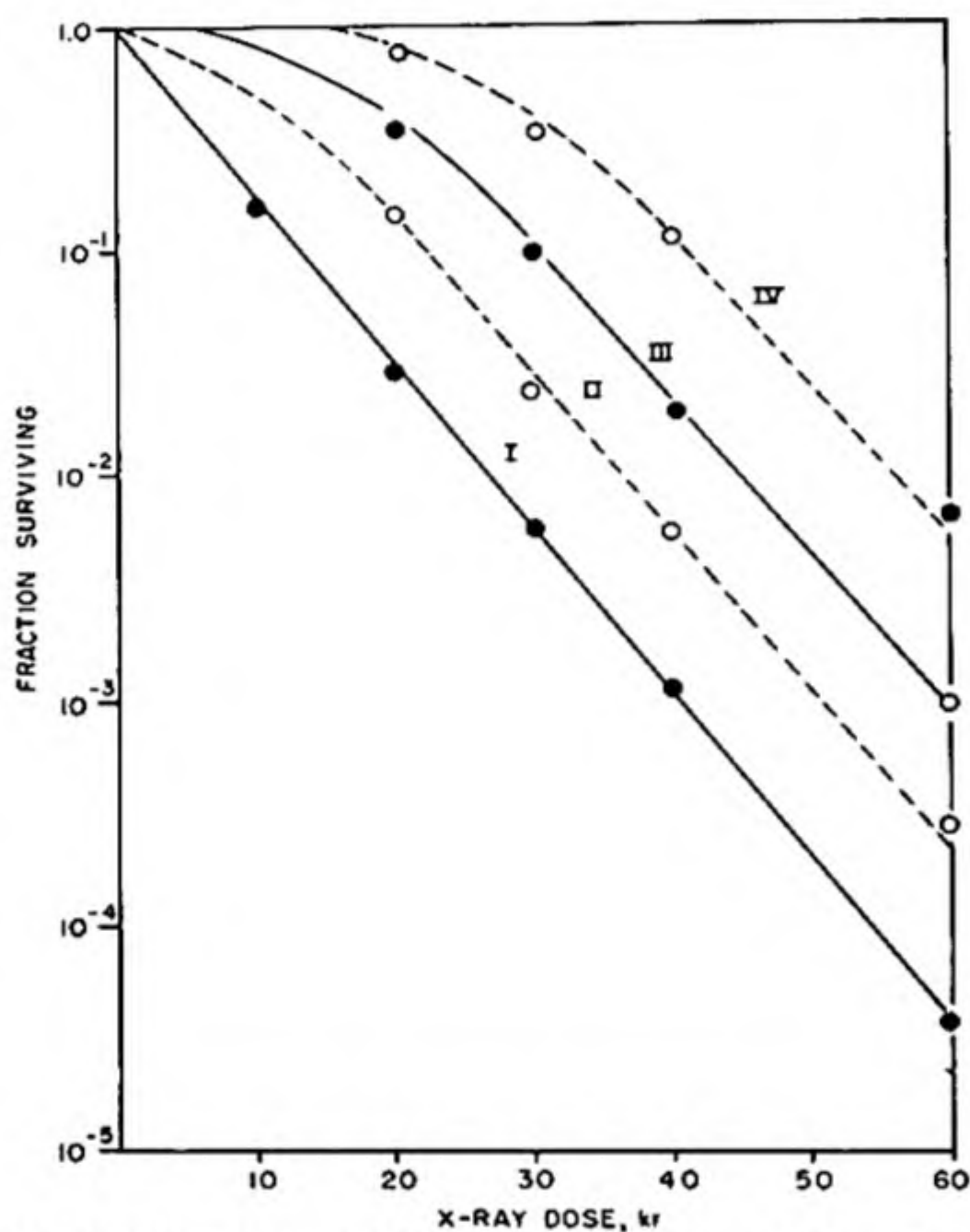


FIG. 10-4. Effect of cultural conditions on X-ray sensitivity of cells irradiated in oxygen-saturated buffer. I, aerobic broth cells; II, anaerobic broth cells; III, aerobic glucose cells; IV, anaerobic glucose cells. (Hollaender, Stapleton, and Martin, 1951.)

following crude classification of compounds so far determined to possess a protective effect:

1. Sulfhydryl compounds: viz., cysteine, mercaptosuccinate, 2,3-dimercaptopropanol (BAL), and 2-(2-mercaptoethoxy)-ethanol.
2. Sodium hydrosulfite ( $\text{Na}_2\text{S}_2\text{O}_4$ ).
3. Alcohols and glycols: viz., methanol, ethanol, isopropanol, propanediol, glycerol, triethylene glycol, and propylene glycol.
4. Metabolic intermediates and products: viz., formate, succinate, pyruvate, fumarate, lactate, and malate.

In general, a preincubation at 37°C is required for maximum protective effect for the metabolic intermediates and alcohols in low concentration



but not for the sulfhydryl compounds and sodium hydrosulfite. Survival ratios up to 500 times those of the control are observed with the optimal concentrations of the various compounds. BAL is roughly twice as efficient as cysteine, which may be the result of its possessing two sulfhydryl groups or of the hydroxyl group also present. Increasing the concentra-

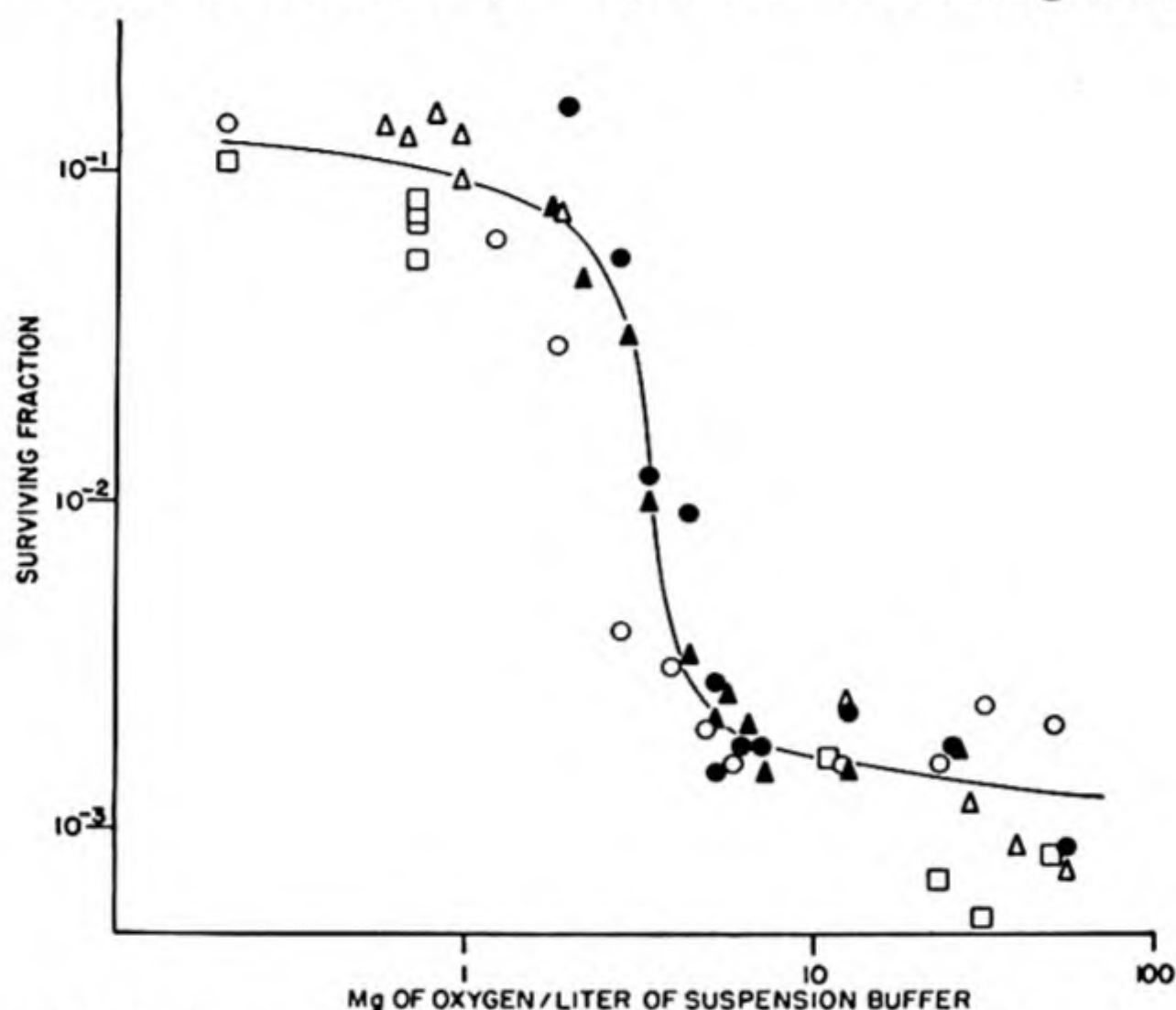


FIG. 10-5. Effect of oxygen concentration on the survival of bacteria exposed to 80,000 v of 250-kvp X rays. (Burnett and Burke, unpublished; see Hollaender and Stapleton, 1953.)

TABLE 10-3. COMPARISON OF THE PROTECTIVE ACTION OF VARIOUS GROUPS OF COMPOUNDS ON *Escherichia coli* B/r (From Hollaender and Stapleton, 1952.)

Class	Lower limiting concentration <sup>a</sup>	Upper limiting concentration <sup>b</sup>	Protection factor with agent <sup>c</sup>	Incubation condition
Sulfhydryl compounds (cysteine and BAL)	0.0025	0.04	3.2-4.0	Without
Inorganic sulfur compounds (sodium hydrosulfite).....	0.00001	0.02	4.0	Without
Alcohols and glycols (ethanol).....	0.005	0.8	2.5-3.0	With
	0.05	3.5	.....	Without
Carboxylic acids (sodium formate).....	0.00005	0.001	3.0	With

<sup>a</sup> The concentration of the compound giving a significant increase in survival.

<sup>b</sup> The lowest concentration required to give the highest survival achieved under indicated conditions.

<sup>c</sup> The protection factor is the ratio of X-ray dose required to inactivate a given fraction of cells in the presence of a protective agent to that required in its absence.

tions of the various compounds results in an increasing protective effect up to a certain level, after which the curves reach a plateau.

Tests for additivity show that compounds within a group are completely additive as long as the total concentration is below the plateau level. There is no increase in protection when increasing amounts of cysteine, for example, are added to optimal or plateau concentrations of BAL. In contrast to this, combinations between groups of compounds do give additive protection when combined at plateau level. Thus all combinations of alcohol, BAL, and sodium hydrosulfite by pairs give additive protection, but combinations of all three give no further increase in protection. This may indicate that the residual killing is the direct effect of the X rays on the cells.

Stapleton, Billen, and Hollaender (1952) have studied the mechanism of the protective action of those compounds which require preincubation for maximum protection. The necessity of preincubation suggests an enzymatic mechanism, and the evidence indicates that only those compounds utilized as oxidative substrates are effective. This was further indicated by tests in which inhibition of respiration was shown to cause a corresponding decrease in protection. In general, respiratory inhibitors had no influence on the degree of protection afforded by sulfhydryl compounds and sodium hydrosulfite although the protective efficacy of cysteine was reduced. Varying degrees of respiratory inhibition with cyanide were accompanied by a corresponding decrease in protection by succinate, suggesting a critical intracellular oxygen concentration. This is also suggested by the sharp increase in protection afforded by an increase in sodium hydrosulfite concentration from about  $2 \times 10^{-4} M$  to  $8 \times 10^{-4} M$ , there being no protection at concentrations lower than  $2 \times 10^{-4} M$ . Studies of cells possessing an active hydrogenase system indicated that neither hydrogen donation nor production of a highly reduced state within the cell is a major mechanism in chemical protection. The respiration studies are compatible with the hypothesis that either enzymatic or nonenzymatic removal of oxygen from around or within the cells is a major mode of protection.

Various hypotheses to explain the mode of action of the different protective substances have been advanced by these workers. In general, these hypotheses involve either a reduction in the yield of toxic radiodecomposition products due to the removal of oxygen from the cellular environment or a competition for highly reactive radiodecomposition products by the protective compound.

Some similar observations have been reported by Thompson *et al.* (1951). They found that the presence of 0.5 per cent pyruvate during irradiation partially protected the bacteria from the lethal and mutagenic effects of X rays and extreme ultraviolet. Addition of pyruvate after irradiation had no effect. Wyss (1951) reported that X or extreme ultra-



violet irradiation of hydrogen-saturated suspensions of *Azotobacter* cells, containing an active hydrogenase, results in less killing and fewer mutations than irradiation of nitrogen- or methane-saturated suspensions. Similar comparisons with *B. anthracis* cells which contain no hydrogenase did not show an effect of hydrogen. Oxygen accentuated the radiation effects with both organisms. It is postulated that pyruvate may exert its effect by reacting with hydrogen peroxide and that the hydrogenase may permit the organisms to destroy oxidizing radicals and peroxide formed by the radiation.

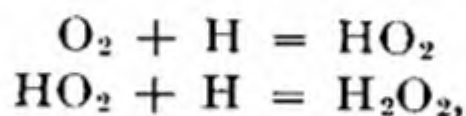
Although speculation about the mechanism of the protective effect of X rays on bacteria may be unprofitable since so little is known concerning this phenomenon, there are a few considerations which may be worth pointing out. First, it seems clear, from the influence of concentration of bacterial cells in liquid suspensions on the inactivation rate and the protective effect exerted by the constituents of nutrient broth and other compounds, that at least part of the inactivation of bacteria by X rays is by an indirect mechanism. Such indirect action of X rays has been shown for bacteriophages by Luria and Exner (1941), for tobacco mosaic virus by Lea *et al.* (1944), and for rabbit papilloma virus by Friedenwald and Anderson (1941). Latarjet and Ephrati (1948) studied the protective effect for bacteriophages of certain amino acids and physiological reducing compounds. In discussing the indirect effects on virus inactivation, Lea (1947) showed that the inactivation dose (37 per cent survival) increases as the nonvirus protein content of the irradiated suspension increases until, at sufficiently high concentrations of nonvirus protein, the inactivation dose essentially equals that for the presumed direct effect as determined by irradiation of dried purified virus protein. He attributed the influence of nonvirus protein to the competition for the active decomposition products formed in water by the X rays. Lea showed that exponential survival curves are obtained by either the direct or indirect action of X rays.

It therefore seems logical to assume that decrease of the oxygen tension or addition of a protective compound is influencing the indirect bactericidal effects of X rays and that the residual killing in the absence of oxygen may result from a direct effect on vital elements of the bacterial cell. Certain complications in the kinetics observed arise on this assumption. Thus, if the presumed direct effect is truly sigmoidal as shown in Fig. 10-3, then the indirect bactericidal effects, when plotted semilogarithmically, must show a very steep negative slope initially which increases as the direct-effect slope decreases and becomes constant when the direct-effect curve becomes semilogarithmically linear. It is difficult to explain such a curve for the indirect effects. If, however, the residual or direct survival curve is exponential as shown by Burnett *et al.* (1951), no difficulty arises. In this connection, it should be pointed out that Lea *et al.*

(1936) observed exponential survival curves when *E. coli* cells were exposed to  $\beta$  and  $\alpha$  particles in dried gelatin films, in which any indirect effects involving radiodecomposition products of water would be minimized.

To attempt any explanation of the observed results, one can only turn to the knowledge of the radiodecomposition of pure water. This extrapolation is questionable since it is known that slight impurities in the water have a profound influence on the results obtained. Hence, greatly different phenomena may occur in the complex chemical milieu within the bacterial cell. However, to attempt any explanation encompassing these considerations seems hopeless in the present state of knowledge (for a brief informative discussion, see Burton, 1951).

The significant observation would seem to be the large effect of oxygen concentration. Since, under irradiation, oxygen is known to be reduced to peroxide in two steps—as shown in the following equation (Allen, 1948)



the chief effect of reducing the oxygen concentration would be to reduce the concentration of peroxide and  $\text{HO}_2$  radicals formed. It would seem therefore that the indirect bactericidal effects of X rays are mediated through either peroxide,  $\text{HO}_2$  radicals, or other unknown radiation products, possibly organic peroxides, depending on oxygen for their formation. It seems equally probable that H atoms and OH radicals are not effective in the indirect action since they are formed independently of oxygen, and, indeed, the presence of oxygen would reduce the number of such radicals free to react with the protoplasmic components involved in the indirect mechanism.

It has been shown that varying the concentration of oxygen has little effect on the frequency of chromosome aberrations produced in *Tradescantia* by  $\alpha$  particles (Giles, Chap. 10, volume I of this series). No similar studies have yet been made with bacteria. This observation, however, is not incompatible with the hypothesis that  $\text{HO}_2$  and/or peroxide are the biologically active water decomposition products, since it is known that detectable amounts of peroxide are formed by irradiation of oxygen-free water with  $\alpha$  particles and negligible quantities are produced by X irradiation. Allen (1948) has shown a general relation between the steady-state hydrogen pressure and hydrogen peroxide concentrations produced in pure water by radiations of different ionization densities, higher concentrations being observed as the ionization density increases.

In summary, the indications are that the indirect effects of X rays are mediated in part by either  $\text{HO}_2$  radicals and/or hydrogen peroxide and that the protective compounds exert their effect either by reducing the oxygen concentration within or immediately surrounding the cells or by



competing for the active products produced by radiation. In addition, Hollaender and coworkers have suggested that certain compounds may give protection by supplying a metabolic intermediate which has been blocked temporarily by the irradiation effect. This possibility would seem to be subject to direct test since, in this case, supplying the particular compound after the irradiation should be effective.

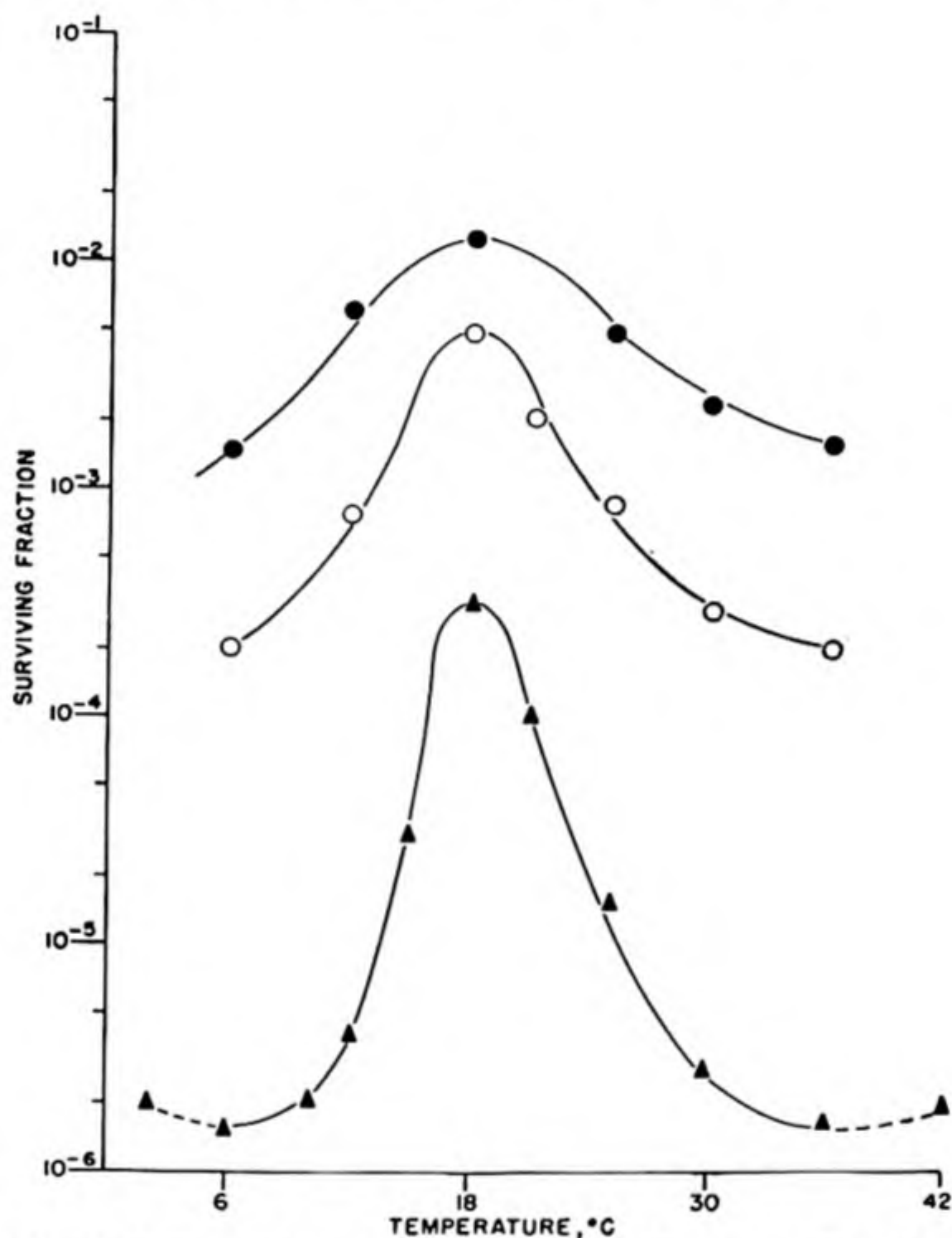


FIG. 10-6. Survival of *E. coli* B/r at several X-ray doses as a function of incubation temperature. Solid circles, 40,000 r; open circles, 60,000 r; triangles, 80,000 r. (Stapleton, Billen, and Hollaender, 1953.)

*Incubation at Suboptimal Temperatures.* Latarjet (1943) has reported a partial recovery of yeast cells subjected to X rays of wave length 1.54 Å when the irradiated cells were held for varying numbers of days at 5°C. No such recovery was observed with bacterial cells similarly tested. Ultraviolet irradiation with 2537 Å wave length was followed by a similar recovery of the yeast cells. Stapleton *et al.* (1953) have observed more than a hundredfold recovery of *E. coli* cells subjected to various doses of X rays when incubated after irradiation at suboptimal temperatures for 24 hours before incubation at 37°C. Figure 10-6 shows typical results.

The optimum temperature for *E. coli* B/r was 18°C, and for the Texas and Crook strains, the optimum temperatures were 26° and 12°C, respectively. The rate of the recovery has been found to be exponential when plotted against time with the length of the exponential phase varying greatly at the different temperatures. An exponential survival curve is found when the surviving fraction following maximal recovery is plotted against X-ray dose, the slope being less steep than that of the curve for the control cells. Studies of the lag phase before cell division and of the rate of recovery suggest that the recovery process is terminated by cell division.

Appreciable recovery does not occur in buffered inorganic salt solutions or in a glucose synthetic medium which supports growth of the cells but does occur in nutrient broth or yeast extract solutions. This eliminates the theory of simple decay of a toxic product resulting from irradiation and indicates that a metabolic process is involved in the recovery. This process could involve either the enzymatic destruction of a toxic product or the synthesis at low temperature of compounds necessary to overcome the potential damage produced by the radiation. The correspondence of the division time with the time required for twofold recovery plus the failure to observe significant recovery in a synthetic medium with a utilizable energy source favor the latter hypothesis. Since it has been shown that irradiation initiates a series of reactions which ultimately are lethal to the cells, Stapleton and coworkers speculate that the optimal temperature of 18°C may be the optimum equilibrium between opposing processes, the recovery process and the unknown processes leading ultimately to inactivation, each with a high temperature coefficient. These significant observations form the first well-substantiated case of recovery of X-irradiated bacterial cells. The important question of the effect of similar recovery on induced mutations is now under investigation.

### ULTRAVIOLET RADIATION

Duggar (1936), Ellis *et al.* (1941), and Loofbourow (1948) have so adequately reviewed the early development of the knowledge of ultraviolet effects on bacteria that it is unnecessary to do so here. Few of the experiments in the fifty years following the original observations of Downes and Blunt (1877) yielded information of a quantitative nature. In addition to the rather qualitative bacteriological methods of demonstrating the bactericidal properties of ultraviolet radiation, the use of nonmonochromatic light sources, the failure to measure intensities, the failure to correct adequately or to control absorption of the incident energy in the suspending medium, and the lack of information concerning the absorption of ultraviolet radiation by bacterial protoplasm were the main factors responsible for the lack of accurate quantitative data. However, research during this period had adequately shown that all bacterial species subjected to ultraviolet radiation of appropriate wave lengths were inacti-





Luckiesh, 1946). Ehrismann and Noethling (1932) report a maximum of sensitivity at 2650 Å for *B. pyocyaneus*, *Micrococcus candidans*, *S. aureus*, a vibrio, and one species of yeast. For *E. coli* they report a maximum sensitivity at 2510 Å and for *Serratia marcescens* at 2804 Å. Duggar and Hollaender (1934a, b) found a maximum of sensitivity at 2650 Å for *S. marcescens*, and Wyckoff (1932), Hollaender and Claus (1936), and Hollaender and Duggar (1936) found the maximum sensitivity of *E. coli* to be at 2650 Å.

An improvement in technique was developed by Hollaender and Claus (1936) who studied the inactivation of *E. coli* cells suspended in a non-absorbing physiological salt solution. This was a modification of the technique used by Duggar and Hollaender (1934a, b). The concentration of bacteria in the suspensions was so great that, except for the small portion of light scattered back into the beam, all the energy incident on the suspension was absorbed. This method reduces the amount of nonspecific absorption which may occur when cells, seeded on the surface of agar plates, are irradiated with ultraviolet. Furthermore, the energy absorbed per bacterium can be calculated directly without the additional source of error involved in estimating absorption coefficients. The bacteria were grown on agar slants and were either suspended in saline solution and irradiated or else washed one or more times before irradiation. Washed suspensions gave a lower MLD than unwashed suspensions, indicating either that washing made the cells more sensitive to radiation or that there was a considerable degree of nonspecific absorption by metabolic products and nutrients in the unwashed suspensions, or both. The action spectra obtained by Hollaender and Claus (1936) showed a sharp maximum at 2650 Å.

The evidence is clear that there is a maximum of bactericidal effectiveness at 2650 Å suggesting that absorption of ultraviolet by nucleic acids or by nucleic acid components is the first step in the reactions resulting ultimately in death of the cell.

Loofbourov (1948) has given the theoretical basis underlying the action-spectrum technique. His analysis showed that the following assumptions are inherent in the action-spectrum method:

1. The biological effect observed is, on the average, attributable to photochemical change in a given number of molecules of an essential substance.

2. The quantum efficiency of the photochemical process is independent of wave length within the region studied.

3. The attenuation of the intensity of radiation before reaching the sensitive substance is either independent of wave lengths or so small in magnitude as to be ignored.

4. The relative absorption of suspected sensitive substances in the cell as a function of wave length either can be estimated with sufficient accu-



racy or can be assumed to be equivalent to the relative extinction coefficients of the suspected substances.

5. The reciprocity law is valid for the times employed in the experiment.

Since in many cases the validity of all these assumptions is difficult or impossible to demonstrate, the action-spectrum technique can be suggestive, but the results must be interpreted with caution. Nevertheless, much useful information has been gained by this technique. Giese (1945) has summarized the data on action spectra of various photobiological effects and has listed seven general types. The observation of the maximum efficiency of bactericidal effects at  $\sim 2600$  Å early focused attention on the purine and pyrimidine constituents of nucleic acids.

#### SHAPE AND SIGNIFICANCE OF SURVIVAL CURVES

Less uniformity in the form of survival curves has been observed with ultraviolet than with ionizing radiations.

Coblentz and Fulton (1924), working with thickly seeded plates of *E. coli*, observed distinctly sigmoidal survival curves with a long threshold exposure before any bactericidal effects were noticed. In some cases the threshold exposure was nearly half the exposure required to kill 90 per cent of the organisms. It is doubtful if the bacteriological techniques employed by these investigators were sensitive enough to detect small amounts of inactivation. Similarly, Gates (1929a, b, 1930) observed sigmoidal survival curves which he interpreted to indicate differences in the sensitivity of individual cells. He computed the extremely skewed distribution necessary to account for the observed curves on this hypothesis.

Baker and Nanavutty (1929) observed survival curves with increasingly steeper slopes when plotted semilogarithmically. They postulated a cumulative action of a toxic substance produced by the ultraviolet radiation as the mechanism.

Wyckoff (1932) obtained exponential survival curves when *E. coli* cells were irradiated on the surface of agar plates with monochromatic ultraviolet. Extending the analysis applied to his results with ionizing radiation, Wyckoff suggested that the exponential curve indicated that the absorption of a single quantum in a vital structure was sufficient to kill the cell, but calculations using absorption data of Gates (1930) showed only one in about 4 million quanta absorbed by the cell was effective. In general, the quantal efficiency of ultraviolet killing of bacteria is very low.

Hollaender and Claus (1936) observed some deviations from exponential killing but, if their data were corrected for the known proportion of double cells determined by microscopic examination, the observed survival could be fitted best by an exponential curve, and their theoretical analysis was based on such a curve. Hercik (1937) observed exponential killing with monochromatic ultraviolet irradiation of *Bacillus megatherium* spores and vegetative cells.

Sharp (1939) presents survival curves for ten species irradiated on agar surfaces with 2537 Å ultraviolet. Although much scatter is evident in the points plotted, in seven of the species there is no systematic deviation from an exponential. The three exceptions were *S. aureus*, *S. albus*, and *B. anthracis*, and clumps or chains of cells could be at least partially responsible.

Lea and Haines (1940), in very careful studies, observed exponential survival curves for *S. marcescens*, *E. coli*, and *B. mesentericus* spores when aqueous suspensions were exposed to 2537 Å ultraviolet.

Witkin (1946, 1947) observed exponential survival curves with *E. coli* B when exposed to 2537 Å ultraviolet but sigmoidal curves with strain B/r. Numerous other workers have made similar observations with these strains. Hence in this case, a bona fide sigmoidal survival curve has been observed since the same bacteriological and physical techniques yield an exponential curve for strain B. Both strains B and B/r are inactivated exponentially by X rays. The explanation for these results is as yet unknown.

In conclusion, it appears that, as with high-energy radiation, exponential or sigmoidal survival curves may be obtained following ultraviolet irradiation, depending on the strain of bacteria and the technique of irradiation including precautions against clumping in the preparation of the bacteria to be irradiated. It should be remembered that it is difficult to determine the precise shape of the killing curve since the low dose range where killing is slight is the most important (see Fano, Chap. 1, volume I of this series).

A number of workers, e.g., Gates (1929a) and Rentschler *et al.* (1941), have attempted to account for the observed survival curves on the basis of variable resistance among the cells making up the population. Others, notably Wyckoff (1932), Hollaender and Claus (1936), and Lea and Haines (1940), interpret the exponential survival curves as indicating that a single quantum is sufficient to kill, sigmoidal survival curves from this point of view being accounted for by the multihit or multitarget theory. Most investigators favor the quantum-hit interpretation since the exponential distribution of resistance necessary to explain cases of exponential survival curves is highly improbable.

#### EFFECT OF INTENSITY

The Bunsen-Roscoe reciprocity law states that the effect of exposure to radiation is a function of the total energy and is independent of intensity and time. Loofbrourow (1948) points out that the reciprocity law is meaningful for photobiological phenomena only when it is restricted to periods of time so short that other kinetic and metabolic activities do not influence the reaction.

Numerous workers have tested the applicability of this law with some-



what conflicting results. Coblenz and Fulton (1924) concluded that the law did not apply, since they observed that a reduction in intensity to one-fiftieth required an increase in exposure of seventy-five- to eightyfold to obtain the same killing effect. These same workers, however, observed no difference in inactivation when the same total energy was given in one continuous dose or in as many as sixteen intermittent doses with varying intervals between. Gates (1929b) studied the effect on an approximately fourfold variation in intensity and observed small differences in survival at the two intensities. The difference in survival at the two intensities diminished as the survival ratio approached zero. Lea and Haines (1940) observed no intensity effect on the inactivation of *B. mesentericus* spores and *E. coli* when the intensity was varied a hundredfold. Koller (1939), in his studies of the lethal effects on air-borne bacteria, observed reciprocity when the intensity of 2537 Å ultraviolet radiation was varied about fifteen hundredfold. The most extreme variations in intensity were those employed by Rentschler *et al.* (1941) who gave the same dose of 2537 Å ultraviolet in periods of time varying from a few microseconds to several minutes. No effect in intensity was observed as long as the total length of exposure was small relative to the generation time of the bacteria. Therefore it appears that the reciprocity law holds approximately for the inactivation of bacteria by ultraviolet radiation if all other factors are held constant.

For very low intensities given over long periods of time, the deviations from reciprocity are greater. Since the energy emitted in the various lines of the mercury-arc spectrum varies widely, it is difficult to avoid differences in intensity of the various wave lengths in action-spectrum studies. However, it would appear that the variation in intensity could have only a very minor influence on the results obtained at the different wave lengths.

#### RELATION OF TEMPERATURE

The early somewhat conflicting results of studies concerned with the relation of temperature to the bactericidal efficiency of ultraviolet have been discussed by Duggar (1936). Bayne-Jones and Van der Lingen (1923) observed temperature coefficients ( $Q_{10}$ ) of 1.06 and 1.04 for the temperature ranges 2°–12°C and 30°–40°C, respectively. Gates (1929b) similarly observed a small temperature coefficient of about 1.1. Exposure at 5° and 37°C had no effect in the studies of Rentschler *et al.* (1941). Heinmets and Taylor (1951) have studied the effect of temperatures as low as –50°C. No pronounced influence of temperature is apparent until –35°C when survival begins to decrease rapidly for a given dose. The low temperature coefficients observed agree well with those expected on the hypothesis that the bactericidal effects of ultraviolet result from a primary, simple photochemical reaction.

## RELATIVE SENSITIVITY OF VARIOUS SPECIES

Comparison of the sensitivity of different species in absolute energy units must be made with caution since there are many factors which can influence the results obtained by different investigators in different laboratories. In Table 10-4 are shown the incident energies of 2537 Å ultraviolet in ergs per square centimeter necessary to inhibit colony formation in 90 per cent of the organisms. This table is taken from Hollaender (1942) and includes some estimates of the sensitivity of *E. coli* B and B/r made from the data of Demerec and Latarjet (1946) and Witkin (1947). These estimates show a striking difference between the two strains of bacteria, one of which is a radiation-resistant mutant of the other. It is of interest that *E. coli* B is the most sensitive strain for which data are available.

## FACTORS INFLUENCING SENSITIVITY

*pH.* Bayne-Jones and Van der Lingen (1923) and Gates (1929b) varied the pH of the medium on which the organisms were irradiated between 4.5 and 9. No appreciable influence of pH on the bactericidal effect was observed although the former workers observed more rapid inactivation below pH 4.6.

*Stage of Growth.* Relatively few investigations have been concerned with the comparative sensitivity of bacteria to ultraviolet at different stages in the growth cycle. Morse and Carter (1949) and Morse (1950) have shown about threefold variation in the DNA content of cells of *E. coli* B and B/r at different stages of the growth cycle. In view of the maximum at 2600 Å in the bactericidal action spectra, it would be surprising if the sensitivity of bacteria remained constant during the growth cycle. Hollaender and Claus (1936) found 7-hr agar slant cultures of *E. coli* to be more resistant to ultraviolet than their standard 15-hr cultures, whereas 10-day-old cultures were more sensitive. Microscopic examination of the 7-hr cultures revealed that 70 per cent of the cells were double, which may account for a large part of the difference in resistance observed. Demerec and Latarjet (1946) observed that growing cells of *E. coli* B/r were more sensitive to 2537 Å ultraviolet than were resting cells. Witkin (1951) reported greatest sensitivity to ultraviolet in the logarithmic phase, greater resistance in the resting stage, and the greatest resistance during the lag phase. These differences in resistance did not parallel differences observed in the mean number of nuclei per cell.

*Relative Sensitivity of Vegetative Cells and Spores.* Few investigators have compared the sensitivity of spores with that of vegetative cells of the same strain. Duggar and Hollaender (1934b) observed *B. subtilis* spores to be about twice as resistant as the vegetative cells. This was confirmed by Hercik (1937) with *B. megatherium* and by Rentschler *et al.* (1941) with *B. subtilis* (Table 10-4).



TABLE 10-4. INCIDENT ENERGIES AT 2537 Å NECESSARY TO INHIBIT COLONY FORMATION IN 90 PER CENT OF THE ORGANISMS

Organism	Energy (ergs/cm <sup>2</sup> ) × 10 <sup>2</sup>	Reference
<i>Bacillus anthracis</i> .....	452	Sharp (1939)
<i>Bacillus megatherium</i> sp.:		
Vegetative cells.....	113	Hercik (1937)
Spores.....	273	
<i>Bacillus subtilis</i> :		
Mixed.....	710	Rentschler <i>et al.</i> (1941)
	600	Koller (1939)
Spores.....	1200	Rentschler <i>et al.</i> (1941)
<i>Corynebacterium diphtheriae</i> .....	337	Sharp (1939)
<i>Eberthella typhosa</i> .....	214	
<i>Micrococcus candidus</i> .....	605	Ehrismann and Noethling (1932)
<i>Micrococcus piltonensis</i> .....	810	Rentschler <i>et al.</i> (1941)
<i>Micrococcus sphaeroides</i> .....	1000	
<i>Neisseria catarrhalis</i> .....	440	
<i>Phytomonas tumefaciens</i> .....	440	
<i>Proteus vulgaris</i> .....	264	
<i>Pseudomonas aeruginosa</i> .....	550	Ehrismann and Noethling (1932)
<i>Pseudomonas fluorescens</i> .....	350	
<i>Sarcina lutea</i> .....	1970	Rentschler <i>et al.</i> (1941)
<i>Serratia marcescens</i> .....	242	
	220	Sharp (1939)
	83	Ehrismann and Noethling (1932)
<i>Shigella paradysenteriae</i> .....	168	Sharp (1939)
<i>Spirillum rubrum</i> .....	440	Rentschler <i>et al.</i> (1941)
<i>Staphylococcus albus</i> .....	184	Sharp (1939)
	330	Rentschler <i>et al.</i> (1941)
	184	
<i>Staphylococcus aureus</i> .....	218	Gates (1929a, 1930)
	260	Sharp (1939)
	495	Ehrismann and Noethling (1932)
<i>Streptococcus hemolyticus</i> .....	216	Sharp (1939)
<i>Streptococcus lactis</i> .....	615	Rentschler <i>et al.</i> (1941)
<i>Streptococcus viridans</i> .....	200	Sharp (1939)
<i>Escherichia coli</i> .....	240 <sup>a</sup>	Gates (1929a, 1930)
	550 <sup>a</sup>	Ehrismann and Noethling (1932)
	640 <sup>a</sup>	Wyckoff (1932)
	211 <sup>b</sup>	Hollaender and Claus (1936)
	500 <sup>a</sup> (est.)	Koller (1939)
	245 <sup>a</sup>	Sharp (1939)
	250 <sup>a</sup>	Rentschler <i>et al.</i> (1941)
<i>Escherichia coli</i> :		
Strain B.....	160, <sup>b,c</sup>	
Strain B/r.....	1200, <sup>b,c</sup>	

<sup>a</sup> Agar surface.<sup>b</sup> Liquid suspension.<sup>c</sup> Estimated from Witkin (1947) and Demerec and Latarjet (1946).

*Moisture Content.* Somewhat conflicting results have been obtained by different investigators who studied the relation between sensitivity to ultraviolet and moisture content of the cells. Thus Koller (1939) and Wells (1940) reported that air-borne *E. coli* are more sensitive than the same organisms floating in liquid suspension. Rentschler and Nagy (1940) reported the same sensitivity for air-borne bacteria and for bacteria exposed on the surface of agar plates. Wells and Wells (1936) and Koller (1939) observed that air-borne bacteria were more resistant to ultraviolet irradiation at high than at low relative humidities. On the other hand, Rentschler and Nagy (1940) found no difference in sensitivity of air-borne bacteria at different relative humidities. A direct relation between X-ray sensitivity of spores of *A. terreus* and their relative water content was observed by Stapleton and Hollaender (1952). Spores containing approximately 25, 42, and 80 per cent moisture had relative sensitivities of 1:1.7:2.4, respectively.

*Enzymatic Constitution.* The enzymatic constitution of bacterial cells is known to depend on the conditions of growth. Thus the enzyme systems of resting bacteria differ from those of growing cells. Similarly, resting cells grown in broth, glucose broth, or synthetic medium differ in enzymatic constitution. Roberts and Aldous (1949) have shown corresponding variation in the ultraviolet sensitivity of cells of *E. coli* B. Resting cells grown in broth (final pH 8) were much less sensitive than resting cells grown in glucose broth (final pH 5.5) or bacteria grown in broth with a final pH of 7.0. Two-hour cultures of *E. coli* B grown in broth and in synthetic medium were more sensitive than cultures of resting cells grown in broth. Not only did the sensitivity change, but the form of the survival curve changed with different growth conditions. In all cases, greater survival was observed when the irradiated cells were assayed on synthetic agar plates than when assayed on nutrient agar plates. Strain B/r, the radiation-resistant mutant of strain B, did not yield similar results.

*Genetic Constitution.* Several references have already been made to *E. coli* B/r isolated by Witkin (1946, 1947). This radiation-resistant mutant was first isolated as one of four surviving colonies from a sample of strain B, which had survived a dose of 1000 ergs/mm<sup>2</sup> of 2537 Å ultraviolet radiation. The mutant strain, which shows greatly increased resistance to ultraviolet and ionizing radiations, has remained stable throughout numerous transfers and has been widely used by many investigators in radiobiological investigations. Witkin found that cells of the parent strain, when subjected to low doses of ultraviolet and plated on agar, formed almost 100 per cent of long filaments. Strain B/r, on the other hand, after a normal lag period of about 1 hr, divided normally. Witkin utilized this observation in a clever double-irradiation technique which permits quantitative estimation of the numbers of resistant cells in



samples of strain B. The method consists in plating samples of the bacteria on agar, irradiating with 50 ergs/mm<sup>2</sup>, and incubating at 37°C for 3 hr. At the end of this time, all the surviving sensitive cells will have formed long filaments. The resistant cells, however, will have divided normally and formed a microcolony of 50–100 cells. The incubated plates are then given a dose of 700 ergs/mm<sup>2</sup>. This exposure will leave from 10 to 20 resistant cells in each microcolony of strain B/r, whereas, if the filaments of sensitive cells behave as single bacteria rather than as chains of bacteria, all the sensitive bacteria will be killed by the second heavy irradiation. This was found to be the case and corresponds to the observation of Lea *et al.* (1937) that the filamentous forms were, like individual cells, killed by a single hit and were equally sensitive to radiation. Uti-

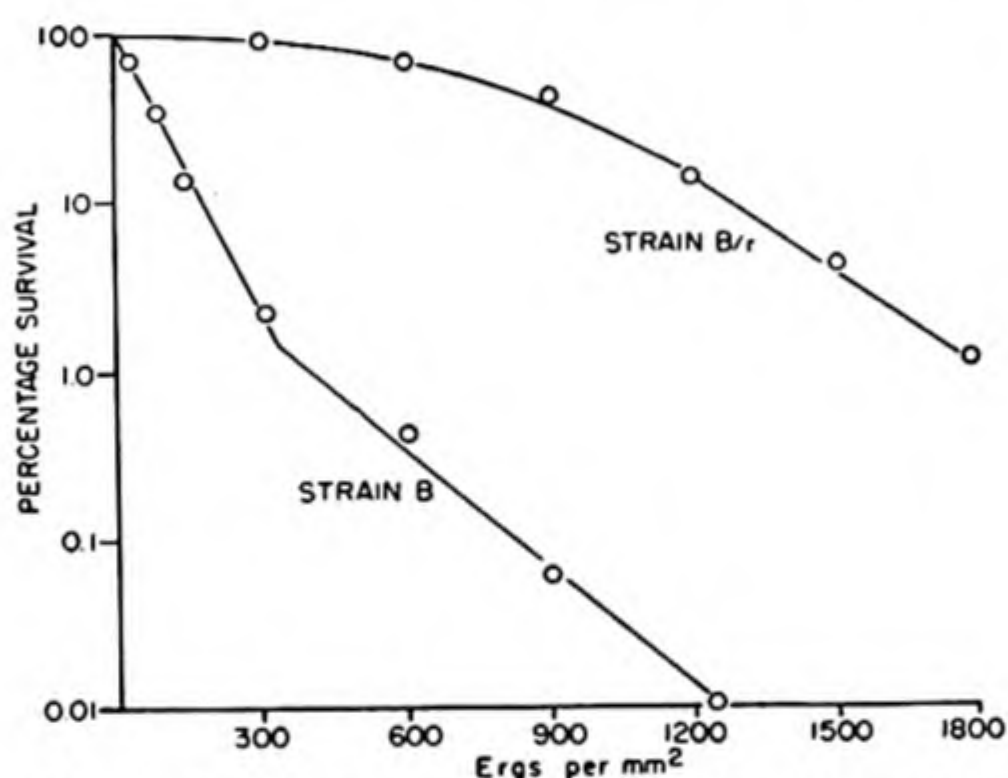


FIG. 10-8. Sensitivity of *E. coli* B and B/r to ultraviolet radiation. (Adapted from Witkin, 1947.)

lizing this quantitative double-irradiation technique, Witkin (1947) demonstrated the mutational origin of the resistant cells and estimated the mutation rate from radiation sensitivity to resistance to be about  $10^{-5}$ . By concurrently testing the resistance of the radiation-resistant mutants to penicillin and sodium sulfathiazole, at least four different types of radiation-resistant mutations were demonstrated. Bryson (1947, 1948) has extended Witkin's observations to include mustard and nitrogen mustard.

Survival curves obtained with *E. coli* B and B/r exposed to 2537 Å ultraviolet radiation are shown in Fig. 10-8. Although strain B is killed exponentially with a change in the slope of the curve at about 1 per cent survival, strain B/r follows a sigmoidal survival curve which, interpreted within the framework of the target theory, would indicate that the mutation to resistance causes a change from a single hit to multiple hits to be necessary for lethality. Both strains are killed exponentially by X rays.

The physiological nature of these genetically determined differences resulting in radiation resistance are as yet unknown.

**Postirradiation Treatment.** Hollaender and Claus (1937) found that holding ultraviolet-irradiated cells in distilled water or physiological salt solution resulted in significant increase in survival. Reference has already been made to the work of Roberts and Aldous (1949) in which they discovered that various postirradiation treatments produced as high as one-hundredfold increases in survival of *E. coli* B which had been exposed to 2537 Å ultraviolet. For example, significantly greater survival was obtained when the irradiated cells were plated on synthetic agar plates than when plated on nutrient agar plates. Furthermore, holding the irradiated cells in fluid media resulted in striking increases in survival. This recovery did not depend to any great extent on the presence of specific factors in the holding fluid; distilled water, saline, synthetic medium, synthetic medium without an energy source, and nutrient broth gave approximately the same results. Varying the pH from 5 to 9 or the concentration of bacteria from  $10^8$  to  $10^3$  per ml likewise had no effect on the extent of recovery. Factors which did influence the recovery in fluid media were the radiation dose and the temperature. As the radiation dose was increased, the rate and final level of recovery attained decreased. The rate of recovery was found to be directly correlated with temperature. When the logarithm of the survival ratio was plotted against dose of ultraviolet for varying periods of recovery, significant changes in the slope and the shape of the survival curves were observed. In Fig. 10-9 are shown the various survival curves obtained. It will be observed that the survival curves change progressively from a concave through a straight-line condition to a convex form as the recovery approaches the maximum possible. Obviously, caution must be exercised in the interpretations placed on the shape of survival curves.

In discussing their results, Roberts and Aldous (1949) point out that very careful control of techniques must be exercised in order to obtain

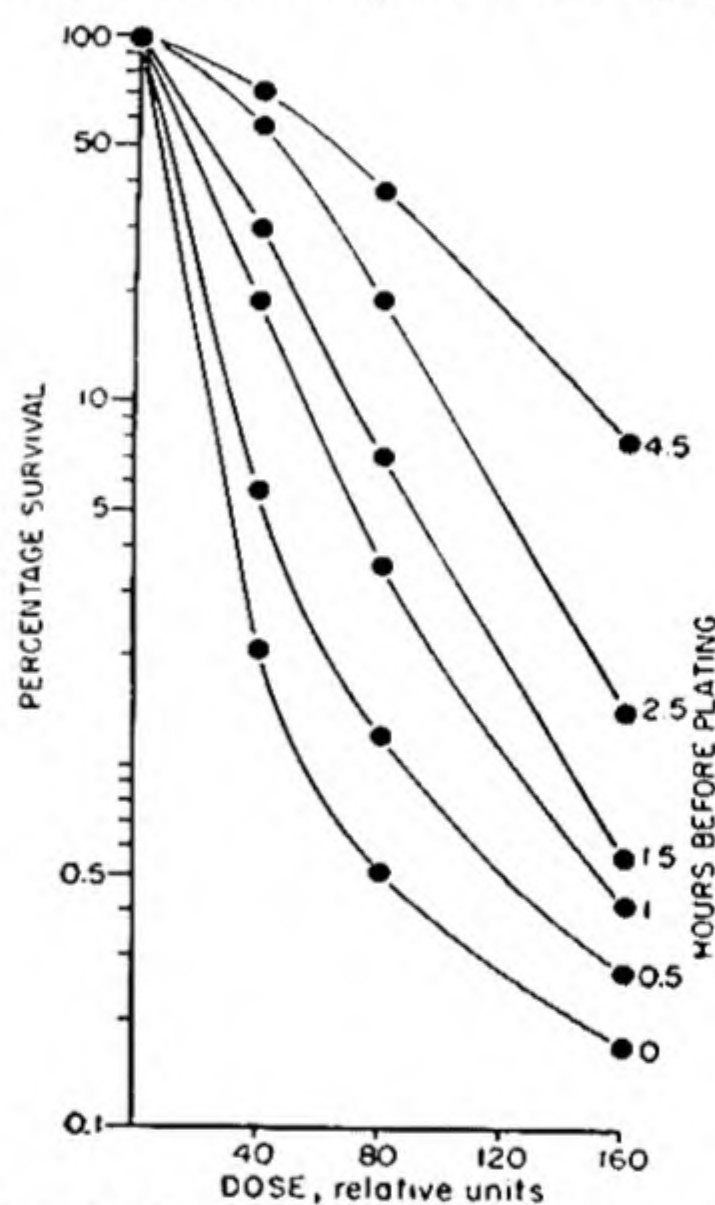


FIG. 10-9. Survival curves of *E. coli* B after 0-4.5 hours of recovery from 2537 Å ultraviolet. (Adapted from Roberts and Aldous, 1949.)



reproducible results. They postulate a poison produced intracellularly by photochemical action which selectively inhibits the mechanism of cell division, one molecule being effective. The recovery is explained by simple exponential decay of the poison. However, they emphasize that since no recovery is observed with strain B/r, a qualitatively different mechanism must be assumed for this strain. Furthermore, since no recovery was observed in either strain with X rays, they point out that the mechanism of X-ray action is qualitatively different from that of ultraviolet.

*Heat Reactivation.* Anderson (1949, 1951a) and Stein and Meutzner (1950) independently observed that incubating ultraviolet-irradiated cells of *E. coli* B at temperatures higher than 37°C results in greater survival. The magnitude of the increased survival is of the same order as that observed when irradiated cells are exposed to visible light (Kelner, 1949b, see section on photoreactivation). The dose-reduction ratios (the ratio of the ultraviolet exposure of cells incubated at 40°C to that for cells incubated at 30°C which produces the same level of survival) for strain B were found by Anderson (1951a) to be a decreasing function of the total dose of radiation, the decrease for heat reactivation being greater than the corresponding decrease for photoreactivation. *E. coli* B/r exhibited only a small heat reactivation, and neither strain showed appreciable heat reactivation following X irradiation. Among ten *E. coli* and seven yeast strains tested by Anderson only two *E. coli* strains were found capable of heat reactivation. It would appear therefore that heat reactivation is not a general phenomenon. Harm and Stein (1952) have shown that the large difference between strain B and its mutant strain B/r in ultraviolet resistance, when the irradiation cells are incubated at 37°C, disappears when the cells are incubated at 44.5°C, equal survival being observed in both strains. Ultraviolet-irradiated cells of strain B remain fully heat reactivable for 1 hr at 37°C (Stein and Harm, 1952). If a longer period elapses before incubation at the reactivating temperature, the amount of reactivation decreases rapidly with no reactivation occurring after 3 hr.

*Photoreactivation.* Among the more significant recent developments is the discovery of the phenomenon of photoreactivation. No exhaustive discussion will be attempted here since Dulbecco reviews the available data in Chap. 12 of this volume.

Although Whitaker (1942) presented conclusive data showing that visible light partially counteracted the effects of ultraviolet radiation in *Fucus* eggs, the present interest in photoreactivation stems largely from work by Kelner (1949a, b). Dulbecco (1949) independently observed photoreactivation of ultraviolet-irradiated bacteriophage adsorbed on sensitive host cells. Kelner (1949a) showed that exposure to visible light subsequent to exposure to ultraviolet radiation would result in as high as 300,000-fold recovery of *Streptomyces griseus* conidia. He later (1949b)

extended his observations to *E. coli* B/r, *Penicillium notatum*, and *Saccharomyces cerevisiae*. By employing a standard photoreactivation treatment which gave maximum recovery, Kelner showed that the effect of photoreactivation was, essentially, to decrease the inactivation rate per unit dose of ultraviolet. Defining the dose-reduction ratio as the ratio of the ultraviolet dose followed by maximum photoreactivation to the ultraviolet dose with no photoreactivating light for the same inactivation, Kelner observed a constant dose-reduction ratio for *E. coli* of about 2.5, which was independent of the survival ratio of the ultraviolet-irradiated suspension. Similar conclusions were reached by Novick and Szilard (1949) who demonstrated that a simple linear relation existed between the survival curves of photoreactivated and nonphotoreactivated ultraviolet-irradiated bacteria. Johnson *et al.* (1950) found that photoreactivation of *E. coli* B following exposure to ultraviolet radiation was independent of the presence of oxygen and that vegetative cells of *Bacillus cereus* showed but little photoreactivation.

The action spectra for photoreactivation of *E. coli* cells and *S. griseus* conidia were investigated by Kelner (1951). For reactivation of *S. griseus* conidia, the effective spectral region extended from 3650 to about 5000 Å with the most effective wave lengths lying near 4360 Å. For *E. coli*, however, the effective wave lengths extended from 3650 to 4700 Å with the most active wave length lying near 3750 Å. Kelner suggests the sharp peak for *S. griseus* conidia at 4360 Å may indicate that porphyrins are involved in photoreactivation.

Heinmets and Taylor (1951) have shown that cells inactivated by ultraviolet at temperatures as low as  $-70^{\circ}\text{C}$  can be photoreactivated when in the liquid state but not in the frozen state. They further showed that cells which have been inactivated by wave lengths of 3000–4000 Å while in the frozen state do not exhibit photoreactivation when in the liquid state, suggesting a qualitatively different mechanism for inactivation by this spectral region.

Photoreactivation seems to be a rather general phenomenon, having been reported for several species of bacteria and bacteriophage (Dulbecco, 1949, 1950), *Paramecium aurelia* (Kimball and Gaither, 1950), *Amblystoma* larvae (Blum and Mathews, 1950), and gametes of the sea urchins, *Arbacia punctulata* (Marshak, 1949; Blum *et al.*, 1950), and *Strongylocentrotus purpuratus* (Wells and Giese, 1950). Bawden and Kleczkowski (1952) observed similar visible-light-induced recovery of ultraviolet-irradiated tobacco necrosis virus inoculated onto French bean leaves and of tomato bushy stunt virus inoculated onto *Nicotiana glutinosa*. No recovery was noted for tobacco mosaic virus inoculated onto *N. glutinosa*. The same workers observed that visible light prevented the ultraviolet-induced necrosis of epidermal cells of *Phaseolus vulgaris* leaves.

Beckhorn (1952) and Kelner (1952) have shown that the extension of



the lag phase following ultraviolet irradiation is photoreversible by visible light. Latarjet (1951) has shown that the induction of active bacteriophage in lysogenic cultures by ultraviolet and also by X rays is similarly photoreversible.

Except for Latarjet's observation (1951), no similar visible-light-induced recovery from the effect of ionizing radiations has been reported, any recovery which may occur being so small in magnitude as to be difficult to demonstrate. This would indicate again that quite different mechanisms are involved in damage by ultraviolet and by ionizing radiations. Furthermore, not all the ultraviolet effects can be reversed by visible light since, in every instance thus far studied, recovery has not been complete. This seems to indicate that more than one mechanism exists by which ultraviolet radiation produces lethal or other effects in cells.

*Other Types of Reactivation.* Working with *E. coli* K12, Monod *et al.* (1949) observed that treatment of the irradiated cells with catalase increased the survival ratio following exposure to 2537 Å ultraviolet. Latarjet and Caldas (1952) have studied catalase restoration in greater detail. Catalase restoration is enhanced by small doses of visible light. The greatest degree of catalase restoration has been observed with *E. coli* K12 and with *B. megatherium* 899, both of which are lysogenic. A non-lysogenic *B. megatherium* strain and *E. coli* B/r show only a slight degree of catalase restoration, whereas *E. coli* B shows none. Neither strains B nor B/r are known to be lysogenic. Catalase restoration requires only small amounts of catalase in contact with the cells for a short period; 5 min is sufficient, and maximum catalase restoration is observed only with rather heavy doses of ultraviolet. The catalase restorability of cells persists for about 2 hr after ultraviolet irradiation and then drops rapidly. No catalase restoration is observed following exposure to X rays.

Lembke *et al.* (1951), in a preliminary note, reported partial reversal of the effects of ultraviolet by treatment of the irradiated cells with certain chemicals. Phenol, glycine, and hydrogen sulfide were effective, whereas chloroform resulted in no reversal.

### EXTREME ULTRAVIOLET RADIATION

Very few data are available concerning the bactericidal or other effects of the extreme ultraviolet (Schumann region) on bacteria. The technical difficulties involved in bactericidal studies in this region are considerable, owing to the absorption of air below 1850 Å. However, Bovie (1916) and Blank and Arnold (1935) have demonstrated the lethal effects of radiation between 1850 and 1100 Å.

Curran and Evans (1938) found that ultraviolet radiations of 2537 Å wave length and in the Schumann region were bactericidal and also that

this wave length sensitized bacterial spores to subsequent exposure to heat. Schumann rays of 1250–1600 Å were several times as effective as 2537 Å in producing this heat sensitization.

#### NEAR-ULTRAVIOLET AND SHORT-VISIBLE RADIATION

Somewhat conflicting results have been obtained by different investigators in studies of the bactericidal action of the near ultraviolet. The upper limit of the bactericidally effective wave lengths as reported by different investigators has ranged from 2967 to as high as 3650 Å. However, there seems little doubt that, although the efficiency of the wave lengths above about 2967 Å decreases greatly corresponding to a similar low absorption of these wave lengths by bacterial cells, exposure to large amounts of radiation in the near-ultraviolet and short-visible regions will produce bactericidal effects as measured by viable count. Duggar (1936) reviews many of the earlier data. The most extensive data are those of Hollaender (1943) who showed a significant reduction in viable count following large exposures to radiation in the region from 3500 to 4900 Å but greatest near 3650 Å. Typical survival curves for the same culture of *E. coli* exposed to 2650 Å and 3500–4900 Å radiation are shown in Fig. 10-10. The survival curve obtained with the latter range of radiation is definitely of a threshold type, no bactericidal effects being noticed until large amounts of energy have been absorbed. Several differences were noticed in the studies of near-ultraviolet and short-visible radiation as compared to the bactericidally effective wave lengths. A temperature coefficient of about 1.7–2.2 for the near ultraviolet contrasts with the value of 1.1 for ultraviolet (Gates, 1929b). The incident energy for a given lethal effect is much greater for the near ultraviolet, and the extension of the lag phase is more pronounced. Reference has already been made to the observation that cells exposed to near ultraviolet are more sensitive to the toxic effect of suspension in saline at 37°C. In Table 10-5 the differences between near-ultraviolet and the bactericidally effective wave lengths are summarized. Hollaender postulates the destruction of an essential cell component, or components, probably generally distributed throughout the cell, which cannot be repaired or replaced from the other cell constituents but can, in many of the cells, be replaced from factors which are present in nutrient broth as the mechanism for the lethal action of near ultraviolet.

The effect of treatment with near-ultraviolet and short-visible radiation in causing photoreversal of ultraviolet effects has already been discussed briefly. In this connection, Heinmets and Taylor (1951) have studied effects of ultraviolet and near-ultraviolet radiation and visible light on frozen bacteria. Photoreactivation was observed to occur only in the liquid phase. Bacteria in the frozen state, whether or not previously exposed to ultraviolet, were inactivated by radiation in the 3000–4000 Å



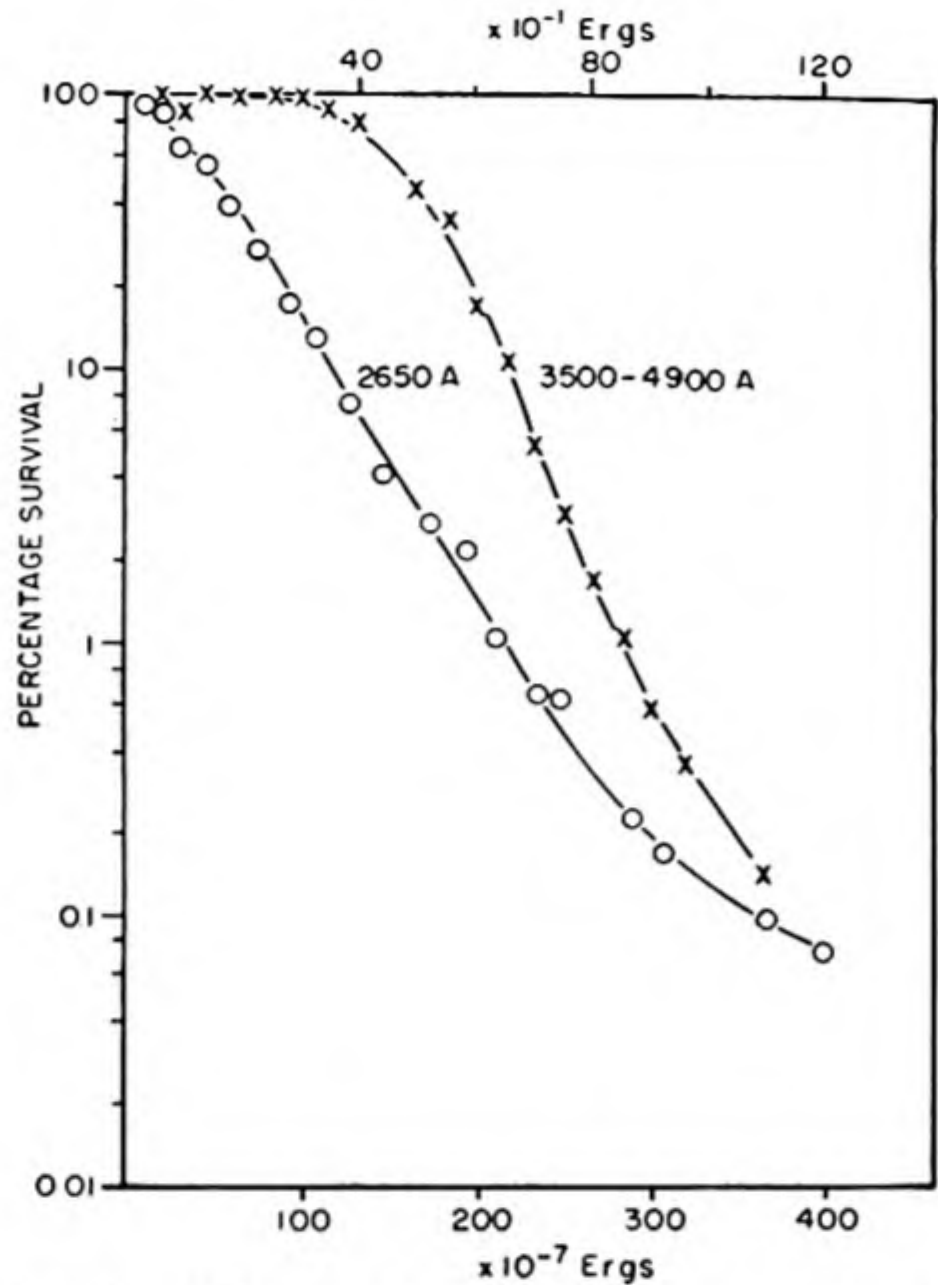


FIG. 10-10. Survival ratio plotted against energy per organism for *E. coli* in liquid suspension. Lower scale, 2650 A; upper scale, 3500–4900 A. (After Hollaender, 1943.)

TABLE 10-5. COMPARISON OF EFFECTS OF THE 3500 TO 4900 A AND THE 2180 TO 2950 A REGIONS  
[From Hollaender (1943)]

	3500–4900 A	2180–2950 A
Shape of killing curve (log survival ratio versus energy)	Threshold type	Approaching straight line
Incident energy for 50 per cent survival ratio (ergs/cm <sup>2</sup> ).....	$2 \times 10^8$	$5 \times 10^2$ – $10^3$
Temperature coefficient.....	1.7–2.2	1.1
Time that sublethal effects appear.....	Before any organisms are killed (in threshold part of killing curve)	After 60–90 per cent of organisms are killed
Extension of retarded growth phase for 10 per cent survival ratio (per cent).. Time that toxicity of certain salt solutions can be recognized	Up to 1000 Immediately after irradiation	50 In 600 min at 32°C
Mutation production.....	None	Mutations produced in fungi and <i>Drosophila</i>

range. Such frozen cells inactivated by 3000–4000 Å radiation showed no photoreactivation in the liquid phase. Thus, Heinmets and Taylor confirm that radiation in this range can cause loss of viability of *E. coli* B cells and that the mechanism of this inactivation is probably quite different from that of ultraviolet radiation of wave length shorter than 3000 Å.

No studies of mutation induction in bacteria by irradiation at wave lengths greater than 3800 Å have been published. Hollaender and Emmons (1946) observed morphological mutations following irradiation of *A. terreus* conidia at wave lengths 2967 and 3130 Å. The energy required was much higher and the maximum proportion of mutants was lower than for 2650 Å ultraviolet. The expected mutagenicity of sunlight, which contains appreciable amounts of energy in the 2900–3150 Å range, was realized experimentally (Hollaender and Emmons, 1946; Hollaender *et al.*, 1946). McAulay and his associates (McAulay and Ford, 1947; McAulay *et al.* 1949; Ford and Kirwan, 1949) report mutations in the fungus, *Chaetomium globosum*, following irradiation with wave lengths of 3354, 3654, and 4047 Å. This work is discussed in more detail later.

In conclusion, it appears that, although irradiation with wave lengths greater than 3000 Å may produce mutations, the mutagenic efficiency is markedly lower than for wave lengths near 2600 Å.

An interesting effect of visible light is photodynamic action, a complex function of visible light and photodynamic dye which requires the presence of oxygen in order to be functional. Duggar (1936) briefly reviewed the earlier work in this field. That permanent hereditary changes in microorganisms can be produced by photodynamic action has been demonstrated by Kaplan. Thus microcolonies of a permanent nature were observed in *S. marcescens* following exposure to visible light in the presence of erythrosin (Kaplan, 1950b). Similarly, reverse mutations in a histidineless strain of *E. coli* (Kaplan, 1950c) and mutations to resistance to coliphage T7 (Kaplan, 1950a) were produced by photodynamic action. Perhaps the most striking effects were obtained with *P. notatum* (Kaplan, 1950d) in which significant increases in colony morphology mutations were observed. Further studies of photodynamic action seem indicated since, as yet, the mechanism and significance of this phenomenon are not very well understood.

#### PHYSIOLOGICAL PROPERTIES OF BACTERIA FOLLOWING IRRADIATION

Surprisingly few investigations have been concerned with the physiology of irradiated bacteria, and much more work is needed in this field.

The observation of filamentous cells following irradiation has been discussed. Chambers and Russ (1912) and Bruynoghe and Mund (1925)



have observed motile cells in irradiated suspensions containing no cells capable of forming visible colonies, indicating that the energy-conversion enzyme systems were still functional. Anderson (1948) has shown that bacteriophage synthesis can occur in ultraviolet-irradiated cells.

The phosphorus uptake and distribution were found to remain normal in irradiated cells (Abelson and Roberts, 1948). Morse and Carter (1949) have studied ribonucleic acid (RNA), DNA, and protein synthesis in normal and irradiated cells of *E. coli* B. Normally, the RNA content per cell increases five- to tenfold, and DNA and nitrogen two- to threefold during the lag phase. After multiplication commences, nucleic acid per cell, especially RNA, decreases. Doses of ultraviolet sufficient to reduce survival to 3–5 per cent produce no delay in the synthesis of nucleic acid. Synthesis of RNA is of normal magnitude, but syntheses of DNA and nitrogen are reduced. Doses sufficient to reduce the viable count to 1 per cent interfere immediately with all syntheses. Relatively little material is synthesized during the normal lag period, after which RNA synthesis appears normal, whereas DNA and nitrogen syntheses are reduced.

Giese (1941) studied the respiration and luminescence of *Achromobacter fischeri* cells after exposure to ultraviolet. Doses, which just inhibited division of most of the cells, permitted a normal respiration rate for 5 hr, followed by a decline in rate. The length of the period of normal respiration was an inverse function of dose, whereas the amount of the decline was proportional to the dose. Very large exposures resulted in an almost immediate large decline in respiration. Luminescence was intermediate in radiation sensitivity between division inhibition and respiration inhibition.

A similar period of apparently normal respiration of X-irradiated *E. coli* B/r cells followed by a decline was observed by Billen *et al.* (1953), who further observed that the duration of the normal period was substrate dependent, being longer on pyruvate and succinate than on glucose. With the Texas strain of *E. coli*, however, an immediate inhibition of respiration was observed on pyruvate. The inhibition of respiration was more pronounced at 37° than at 26°C.

Brandt *et al.* (1951) found that ultraviolet radiation inhibited the adaptive formation of galactozymase in *Saccharomyces cerevisiae*. Doses which completely inhibited synthesis of the enzyme had little effect on preformed enzyme. Exposure to 4850 r of X rays did not inhibit formation of galactozymase, even though division was inhibited in 90 per cent of the irradiated cells.

In similar studies, Billen and Lichstein (1952) observed that increasing doses of X rays caused an increasing inhibition of the adaptive formation of formic hydrogenlyase in *E. coli*. Doses of 60,000–90,000 r had no measurable effect on preformed enzyme but completely inhibited the adaptive synthesis of formic hydrogenlyase. Although the inhibition of

enzyme formation was proportional to dose for exposures less than 60,000 r, the amount of enzyme synthesized in such irradiated cell suspensions was much greater than could be attributed to the cells still capable of forming colonies. These studies suggest that inhibition of enzyme synthesis may be more important in the bactericidal effect of radiation than inactivation of enzyme molecules already present in the cells, as suggested by Dale (1940, 1942).

It appears that irradiated cells rendered incapable of forming visible colonies are still able to perform many if not most of the normal metabolic functions. Complex processes such as growth and phage synthesis would seem to indicate that no gross disturbances of metabolism result from irradiation and that the cell membrane remains essentially intact. In this latter connection, however, Loofbourow and associates (for review, see Loofbourow, 1948) have shown the accumulation in the suspending medium of growth-promoting factors from irradiated cells, and Billen (personal communication) has shown a leakage of adenosinetriphosphate from *E. coli* cells following exposure to X radiation.

The bactericidal effects of radiation have long been recognized more as a specific inhibition of cell division than as a general inhibition of metabolism, and the results discussed indicate greater radiosensitivity of cell division as compared to the radiosensitivity of respiration and of certain synthetic processes. Morse and Carter's investigations of nucleic acid synthesis are an example of further studies of specific metabolic functions intimately related to cell division which are needed to elucidate the particular metabolic processes involved in the inactivation of bacterial cells by radiations.

### SUBLETHAL EFFECTS OF RADIATION

The genetic changes are the most widely studied sublethal effects of radiations. Before considering the genetic phenomena, however, some other sublethal effects will be considered.

The extension of the lag phase observed by numerous workers has been studied in some detail by Hollaender and Duggar (1938). Working with *E. coli*, they had observed a delay in the appearance of colonies following irradiation with ultraviolet of 2650 Å wave length. During their study of the delay in growth of irradiated organisms in liquid cultures, they observed a second effect, an apparent initial increase in the number of cells. Thus, although the control suspensions showed no increase in numbers of cells for the first 2 hr of incubation, the irradiated suspensions showed a significant increase. The apparent early increase in numbers of cells did not eliminate the extended lag phase, the duration of which was estimated by extrapolating the logarithmic portion of the growth curve back to zero growth. The extension of the lag phase is evident with survivals as high as 70 per cent, whereas the apparent initial increase does



not appear until doses resulting in 20 per cent survival have been given and is more pronounced with doses resulting in less than 8 per cent survival. Kelner (1952) and Beckhorn (1952) have shown that the extension of the lag phase is photoreversible by visible light to about the same degree as the bactericidal effects.

### BACTERIAL GENETICS

Although very little was known concerning bacterial genetics prior to about 1940, the development of knowledge in this field has been rapid since that time. Inasmuch as excellent reviews are available (Lederberg, 1948, 1951; Braun, 1947; Luria, 1947), no extended discussion is necessary here.

The methods of classical Mendelian genetics are not applicable to genetic analysis since, in general, bacteria reproduce asexually. Instead, the chief tool of the bacterial geneticist must be an analysis of mutation. The case for the genic nature of heredity in bacteria, therefore, must rest largely on a number of analogies to the behavior of genes in higher, sexually reproducing organisms. That the fundamental unit of inheritance in bacteria is the gene is strongly indicated by the repeated observations of the permanence of mutated characteristics, the spontaneous occurrence of these characteristics independent of specific environmental stimuli at definite rates, the independence of different mutations in the same organism, mutation inducibility by mutagenic agents known to be effective in higher organisms, the reversibility of mutations, and the occurrence of mutations with physiological effects similar to known gene mutations in sexually reproducing organisms.

Luria and Delbrück (1943) showed that the numbers of mutant cells in parallel cultures of bacteria should follow a clonal rather than a random sampling distribution if they resulted from preadaptive mutation. Such was found to be the case for bacteriophage-resistant mutants, and their so-called "fluctuation" test has since been applied successfully to other types of bacterial mutations. More direct demonstrations of the mutational origin of bacterial variants have been given by Newcombe (1949) and Lederberg and Lederberg (1952).

In addition, Lederberg's thorough analysis (Lederberg, 1947, 1949; Lederberg *et al.*, 1951) of the phenomenon of recombination in *E. coli* K12 (Tatum and Lederberg, 1947) shows the physical basis of inheritance in bacteria to be essentially similar to that in higher organisms. Haas *et al.* (1948) have shown that sublethal doses of ultraviolet increase the frequency of sexual recombination in strain K12. Although the actual fusion of cells of two mutant strains to form heterozygotes has not been demonstrated, Zelle and Lederberg (1951), by means of single-cell studies

on heterozygous strains, have shown convincingly that individual cells contain the genetic potentialities of the two parent strains.

Cytologically, it is known that bacteria possess Feulgen-positive bodies which Robinow (1945) considers as chromosomes. Furthermore, the number of such bodies is known to vary from one to four or more per cell depending on the species, the stage of the growth cycle, and the stage in the division cycle of the individual bacterium observed. Observations indicating a genetic role for the so-called "chromatinic bodies" are just beginning to appear. Lederberg *et al.* (1951) have made comparative cytological studies of *E. coli* cells known to be haploid or diploid from genetic evidence. Perhaps the strongest evidence indicating an actual genetic function for these structures is that of Witkin (1951) who found a correspondence between the average number of chromatinic bodies per cell and the size of nonlactose-fermenting mutant sectors in colonies of *E. coli*. Zelle and Lederberg (1951, and unpublished observations) have genetic evidence that individual cells can be multinucleate, since a single cell has been observed to divide and form a diploid and haploid cell, the differentiation being based on subsequent genetic behavior. The fact that bacterial cells do not necessarily have a constant nuclear constitution has an important bearing on the mechanism of bactericidal effects of radiation as well as an obvious relation to genetic phenomena in bacteria.

If it is granted that the basic unit of inheritance in bacteria is the gene and that gene mutations are responsible for the observed heritable variations, bacteria become a very valuable experimental tool because of the relative ease of making quantitative studies of both spontaneous and induced mutations. Systems of bacterial mutations which are particularly valuable in such quantitative studies include mutations to resistance to certain antibacterial agents such as bacteriophages, antibiotics, radiation, and metabolic inhibitors; reverse mutations of biochemical mutations in which the ability to carry out certain syntheses has been lost; and mutations affecting the fermentation reactions.

It has generally been assumed that the kinds of viable mutations observed following irradiation do not differ from those which occur spontaneously, and that irradiation merely increases the rate of occurrence. Bryson and Davidson (1951) have studied a series of independently occurring, spontaneous, and ultraviolet-induced mutations to resistance to T1 bacteriophage in *E. coli* B/r. The mutants were analyzed for resistance to the other phages of the T series, for the tryptophane requirement which Anderson (1946) has shown generally accompanies spontaneous mutation to resistance to phage T1, and for other biochemical requirements. Significant differences were observed in the proportions of T1-resistant and T1- and T5-resistant mutations in the spontaneous and irradiated series. Similarly, the frequency of tryptophane requirement



was significantly lower in the irradiated series. No complex phage-resistant mutants were observed in the irradiated series, and, although no other biochemical requirements were observed in the spontaneous mutants, five biochemical requirements other than tryptophane were noted in the 114 radiation-induced mutants studied. These are the most critical data bearing on this important question and indicate that radiation-induced mutations may be qualitatively different from those that occur spontaneously.

### RADIATION-INDUCED MUTATIONS IN BACTERIA

Stable heritable variation in colony morphology, cellular morphology, and pathogenicity were observed by Henri (1914) among the surviving cells of *B. anthracis* cultures exposed to ultraviolet radiation. In addition, unstable variants occurred which reverted to the normal type. Stable variants were produced in numerous experiments and appear to have been bona fide mutations although, of course, the underlying cause was not proved to be genic.

Shortly after Muller's announcement of the mutagenic effect of X rays in *Drosophila* (1927), Rice and Reed (1931) studied rough-to-smooth variation in a bovine strain of *Mycobacterium tuberculosis* irradiated with 88-kvp X rays. Their results are hard to evaluate in that the rough-to-smooth change occurs spontaneously and the smooth forms are quite unstable. Haberman and Ellsworth (1940) qualitatively demonstrated that X rays increased the so-called "dissociative" changes in *S. aureus* and *S. marcescens*.

The first quantitative study of X-ray-induced mutations in bacteria was that by Lincoln and Gowen (1942) in which various colonial characteristics of *Phytomonas stewartii* were utilized. The data show clearly an increase in the frequency of mutations among the cells surviving X irradiation, and the rate of mutation for the three major characteristics studied was about  $3.7 \times 10^{-8}$  per character per roentgen. Croland (1943) observed a hundredfold increase in mutants per  $10^8$  cells in studies of the succinate-minus to succinate-positive mutation in *Moraxella lwoffii* with X rays. He observed an increase in the absolute number of mutants as well as in the proportion of mutants among the survivors.

Biochemical mutants that affect specific syntheses were reported by Roepke *et al.* (1944) and Gray and Tatum (1944) following X-ray treatment. Since that time, numerous investigators have succeeded in isolating biochemical mutations in various species of bacteria.

The work of Demerec and Latarjet (1946) and Anderson (1951b), who have published the most precise quantitative studies of induced mutations in bacteria, will be discussed later.

Burkholder and Giles (1947) induced biochemical mutations with ultraviolet and X-ray treatment of *B. subtilis* spores, and Devi *et al.*

(1951) isolated mutations affecting the nutritional requirements of *Aerobacter aerogenes* when the cells were exposed to X rays in the dry state.

#### DELAYED EXPRESSION OF INDUCED MUTATIONS

Demerec (1946) observed a delay in the phenotypic expression of ultraviolet-induced mutations to resistance to T1 bacteriophage. Only about 1 per cent of the induced phage-resistant mutations were observed as mutants when irradiated cells were plated upon the surface of agar plates containing an excess of phage. The peak expression of induced mutations occurred after one or two generations of growth, and induced mutations were observed to continue to appear until 12 or 13 generations of growth had occurred. The technique which made these observations possible consisted in infecting the bacteria, after varying periods of growth on agar plates, by exposing the plates to an aerosol of the bacteriophage, a method which does not disturb the distribution of cells on the plates. Later, Newcombe (1948) observed a similar delay in the expression of spontaneous mutations to phage resistance although the appearance of mutations did not continue through as many generations of growth. Newcombe and Scott (1949), in studies of the factors responsible for the delayed appearance of radiation-induced mutants, concluded that a phenotypic lag in expression of mutations, similar to that occurring in spontaneous mutations, plus a variable delay in the onset of division of the irradiated cells are responsible.

Demerec, Dollinger, and Flint (1951) found quite different patterns in the phenotypic expression of induced phage-resistant and streptomycin-resistant mutations. Witkin (1951) employed a double-screening technique to obtain evidence that induced mutations in *E. coli* were sometimes expressed in only part of a clone derived from a surviving irradiated cell. These results indicate that nuclear segregation, a physiological lag, and a variable onset of division are not sufficient to explain the delay in expression of radiation-induced mutations.

A complete discussion of delayed expression of mutations is beyond the scope of this chapter. It is obvious, however, that this delay in expression is an important factor which must be considered in quantitative studies of radiation-induced mutations in bacteria.

#### QUANTITATIVE STUDIES OF RADIATION-INDUCED MUTATIONS

In an important paper, Demerec and Latarjet (1946) have reported studies on ultraviolet- and X-ray-induced mutations to resistance to T1 bacteriophage in *E. coli* B and B/r. Both zero-point mutations (i.e., mutations expressed immediately) and end-point mutations (i.e., total mutations induced including those expressed immediately and after a delay) were assayed. In Fig. 10-11 are shown the results for ultraviolet irradiation of strain B/r.



For zero-point mutations the rate of increase in the number of mutations with increasing dose exceeds an exponential function of dose. The frequency of mutations increases until doses causing about  $10^{-5}$  survival are exceeded, at which time a plateau followed by a slight decline is observed. A similar decline in mutations at high doses has been observed by Hollaender and Emmons (1939a, 1941; Emmons and Hollaender, 1939) with the fungus *Trichophyton mentagrophytes*, Hollaender, Raper, and Coghill (1945) with *A. terreus*, and Hollaender . . . Demerec (1945) for *Neurospora*. No good explanation exists for the occurrence of this maximum in the mutation-dose relation.

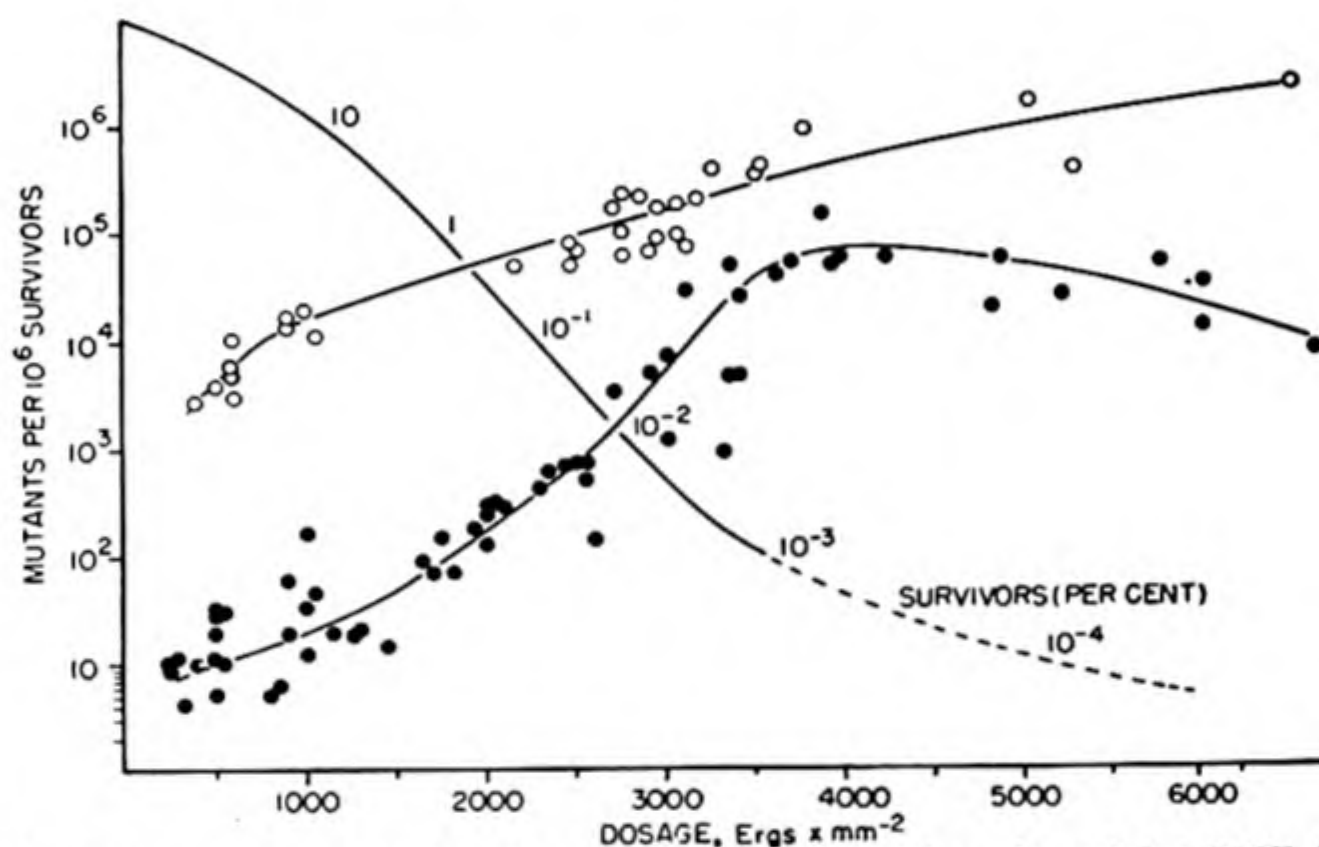


FIG. 10-11. Zero-point and end-point mutants induced by ultraviolet 2537 Å in *E. coli* B/r, and ultraviolet survival curve of B/r (semilogarithmic plot). Solid circles, zero-point mutants; open circles, end-point mutants. (Adapted from Demerec and Latarjet, 1946.)

For end-point mutations, Demerec and Latarjet (1946) observed a rapid increase in mutations at low doses, but as the dose continued to increase the rate of increase in mutations decreased and continued more or less exponentially to the highest doses tested, no maximum being observed for the end-point mutations. The frequency of end-point mutations was higher at all doses than that of zero-point mutations.

With X rays, essentially similar results were obtained for both zero-point and end-point mutations, the latter being approximately 200 times as frequent at all doses. The mutation-dose curves were approximately linear, which is compatible with a one-hit mechanism, whereas the much-greater-than-linear increase in mutations with increasing dose observed for ultraviolet irradiation indicates a multiple-hit relation.

In comparative experiments with strains B and B/r, nearly identical phage-resistance mutation rates were observed at a given dose of either ultraviolet or X rays even though the inactivation of strain B was much

greater. This observation has important implications with respect to the mechanism of the lethal action of radiation. On the basis of energy absorbed per bacterium, X rays were found to be approximately 200 times as efficient in producing inactivation and about ten times as efficient in the production of mutations.

In later studies, Demerec (1949, 1951) observed a linear increase in reverse mutations of the SD-4 streptomycin-dependent mutant of strain B/r (Bertani, 1951) with both X and ultraviolet irradiation. With this mutation system, no zero-point mutations occur since no mutations are expressed until at least one division has occurred.

In this connection, Grigg (1952) has shown that, in quantitative studies of reverse mutations of biochemical mutations in *Neurospora* conidia, precautions must be taken to ensure that the observed reversions are actually mutations and are not already present in the irradiated culture. He found that large numbers of mutant conidia which require a particular growth factor inhibit the growth of a known smaller number of wild-type cells when plated on minimal medium. He suggested that, in induced-reverse-mutation experiments, the radiation merely kills enough of the growth-factor-requiring cells to remove the inhibition, thus permitting spontaneous reverse mutations already present in the culture to develop. This was demonstrated in tests in which the apparent proportion of wild-type nuclei increased tenfold when the culture was plated at low dilutions. Somewhat similar observations have been reported by Bryson (1950) with streptomycin-resistant mutants of *E. coli*.

Still another type of mutation-dose relation has been observed by Newcombe and Whitehead (1951) in studies of color-response mutants of *E. coli* B/r on mannitol-tetrazolium agar. With this system the frequency of mutation rises very rapidly with ultraviolet doses below 500 ergs/mm<sup>2</sup> and is nearly constant for doses of 1000–5000 ergs/mm<sup>2</sup>. No decline in the percentage of mutations is observed at the higher doses.

Extensive quantitative studies of reverse mutations of the same SD-4 mutant of strain B/r used by Demerec (1949, 1951) and of a purineless mutant of the same strain were made by Anderson (1951b). The chief results of his experiments, which were designed to test whether the oxygen concentration at the time of X irradiation had a parallel influence on mutagenesis and inactivation, are shown in Figs. 10-12 and 13. Survival curves for the two mutant strains were nearly identical. However, greatly different response curves were obtained for the two reverse mutations. Thus Fig. 10-12 shows that, for the streptomycin-dependent strain, a linear relation exists between induced mutations and dose and that only a slightly higher rate of induced mutation was observed when the organisms were irradiated in the presence of oxygen. For the purineless strain, an approximately exponential increase in mutation rate was observed with increasing dose, the frequency of mutations observed for a



given dose of radiation being much greater in the presence of oxygen than in its absence. The same mutation data are plotted against survival ratio in Fig. 10-13 to illustrate more clearly that, for the streptomycin-dependent strain, the frequency of back mutation is greater for a given survival level when the cells were irradiated in the absence of oxygen and

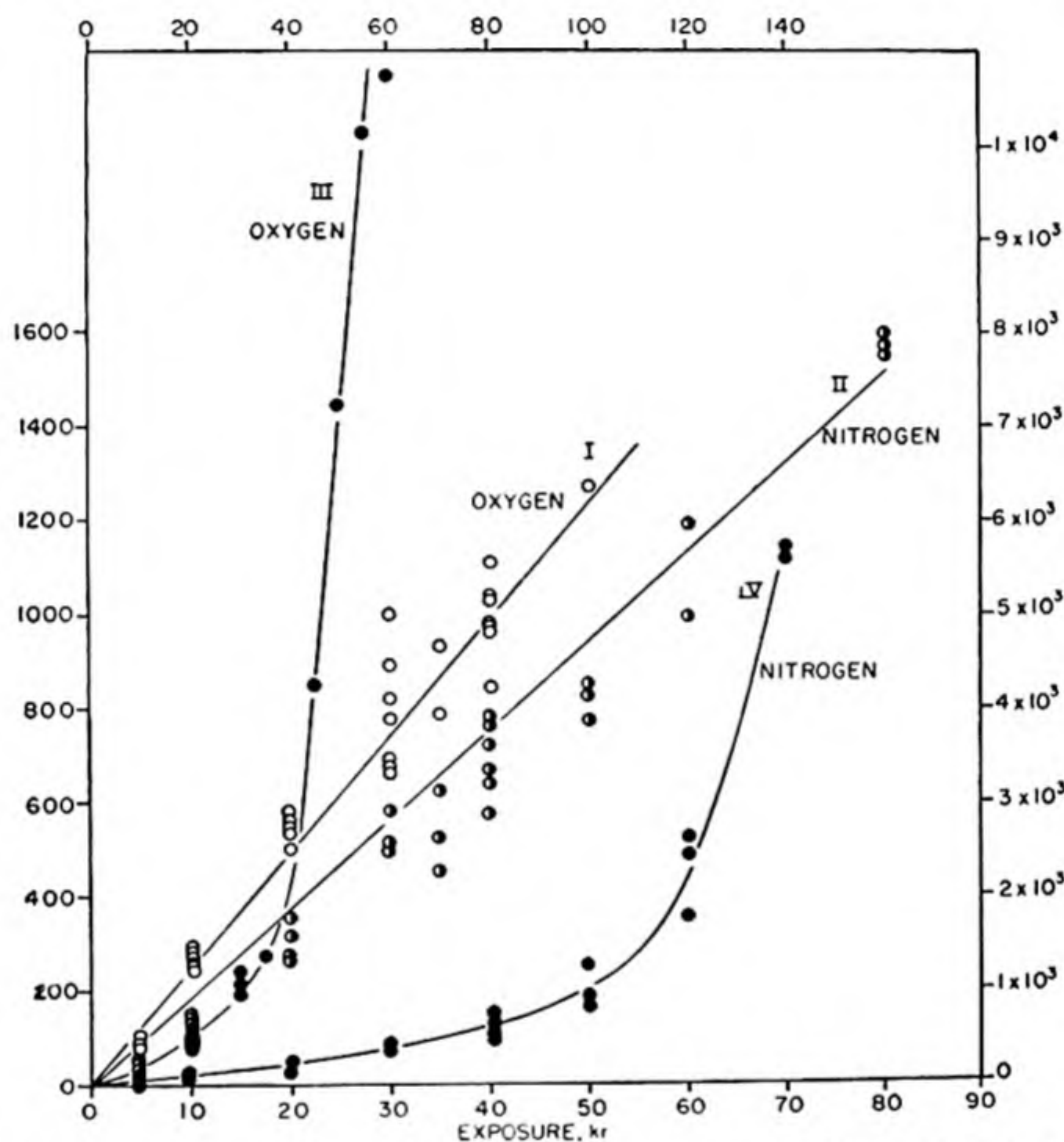


FIG. 10-12. X-ray induction of back mutations as a function of kiloroentgens of exposure. Left and bottom scales, comparison of back-mutation rates to streptomycin nondependence of the streptomycin-dependent strain when irradiated in oxygen (curve I) or in nitrogen (curve II). Right and top scales, comparison of the back-mutation rates of the purineless strain to purine nondependence when irradiated in oxygen (curve III) or in nitrogen (curve IV). (Anderson, 1951b.)

hence had received approximately two and one-half times as much incident energy as the cells irradiated in oxygen. For the purineless back mutation, the mutation-survival curves are identical for cells irradiated in the presence and in the absence of oxygen. These results are significant in indicating that greatly different quantitative relations may exist between the induced-mutation rates and radiation dosage for different mutation systems. Furthermore, although the oxygen-sensitive indirect mechanism which influences inactivation of both strains appears to have

a quantitatively equal effect on the purineless reverse mutation, it does not seem to be a major factor in the induction of streptomycin-dependent reverse mutation.

Demerco *et al.* (1952) have studied the reverse-mutation response of 27

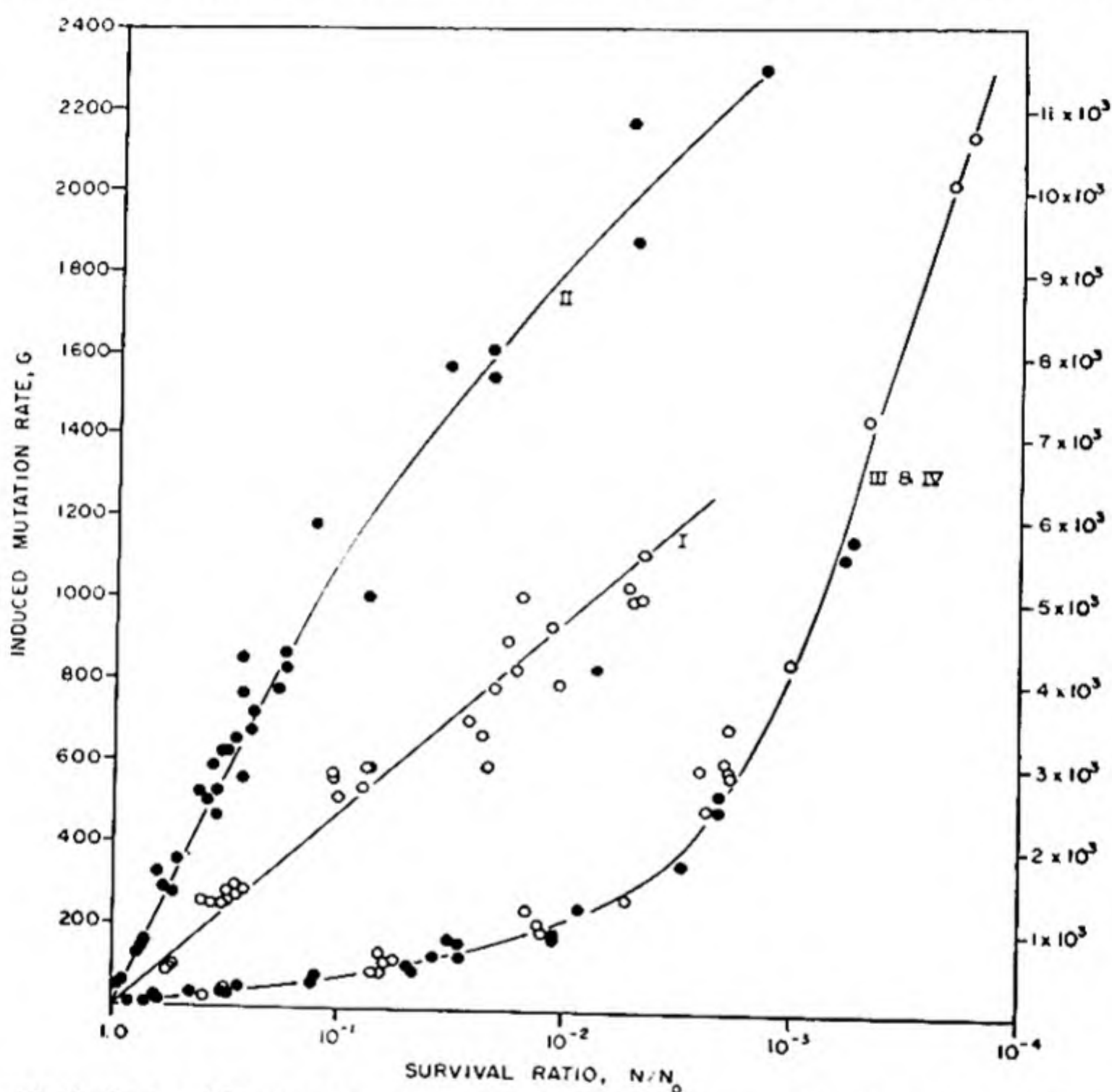


FIG. 10-13. Rate of X-ray induction of back mutations as a function of survival ratio. Left scale, comparison of the back-mutation rates to streptomycin nondependence of the streptomycin-dependent strain irradiated in oxygen (curve I) or in nitrogen (curve II). Right scale, comparison of the back-mutation rates to purine nondependence of the purineless strain irradiated in oxygen (curve III) or in nitrogen (curve IV). (Anderson, 1951b.)

mutant strains of *E. coli* to a variety of mutagenic treatments. The most striking observation was that in four of the strains the spontaneous rate of back mutation could not be increased by any of the mutagenic treatments, which included X and ultraviolet radiation, manganous chloride, and  $\beta$ -propiolactone. In addition, the quantitative response of the other strains to ultraviolet and manganous chloride varied differentially.

It seems clear that all bacterial mutations do not exhibit the same



quantitative relations to radiation or other mutagens and that some may not respond to radiation or other mutagens at all. That such divergent results should be obtained with the first few specific mutations adequately studied indicates that caution should be exercised in generalizing, as regards the mutation-dose relation, and that equal caution should be used in interpreting radiation-induced mutation rates which are the sum of the mutation rates of an unknown number of loci.

#### PHOTOREACTIVATION OF ULTRAVIOLET MUTAGENIC EFFECTS

Although this subject is covered more completely in Chap. 12 by Dulbecco, a brief discussion seems appropriate here. Since photoreactivation indicates an indirect mechanism for a major proportion of the ultraviolet bactericidal effects, it is of interest to know if the same indirect mechanisms are involved in the production of mutations following ultraviolet irradiation. Kelner (1949b) and Novick and Szilard (1949) were the first to study this question and, although Kelner's results were somewhat inconclusive, Novick and Szilard observed a reduction in mutagenic effects to a degree corresponding roughly with the reduction in the lethal effects. Similar observations have been reported for *Paramecium aurelia* (Kimball and Gaither, 1950; Kimball, 1950), *Neurospora* (Goodgal, 1950; Brown, 1951), *Penicillium chrysogenum* (Roegner, 1951), and for the polar cap cells of *Drosophila* (Meyer, 1951). Roegner's results are of interest in that the mutation-dosage relation exhibited a maximum, as discussed earlier. When the photoreactivation treatment followed high doses of ultraviolet, i.e., on the descending portion of the curve beyond the maximum, Roegner observed an increase in the frequency of mutation, but at lower ultraviolet doses in the ascending portion of the curve, photoreactivation resulted in a decreased proportion of mutations.

The most extensive studies are those of Newcombe and Whitehead (1951) with the color-response mutations of *E. coli* B/r on mannitol-tetrazolium agar. A relatively constant proportion of mutations was observed for ultraviolet doses greater than 1000 ergs/mm<sup>2</sup>. Photoreactivation of low doses of radiation resulted in a dose-reduction factor of 5 which is greater than that observed for the bactericidal effects. For very large doses of ultraviolet, no effect of photoreactivation in lowering the mutation frequency was observed. To explain their results, Newcombe and Whitehead postulate that both a photostable and photosensitive mutagen poison are produced by the ultraviolet and that the plateau in the mutation-dose curve is due to a common limiting step in the reactions by which both of these mutagen poisons cause mutations. Thus, at high doses, a sufficient amount of the photostable mutagen poison is produced to cause a maximum mutation effect, and no photoreactivation is possible.

In studies of ultraviolet-induced mutations from streptomycin dependence to independence in the SD-4 strain of *E. coli*, Beckhorn (1951)

observed a decrease in induced mutation rates following photoreactivation at all ultraviolet doses. In this case, however, there is no maximum or plateau in the mutation-dose curve.

#### ULTRAVIOLET ACTION SPECTRUM OF MUTATION INDUCTION

Action spectra of mutation induction with maxima in the neighborhood of 2600–2650 Å have been reported by Knapp *et al.* (1939) for *Sphacrocarpus donnelli* sperm; Hollaender and Emmons (1939a, b, 1941) and Emmons and Hollaender (1939) for *Trichophyton mentagrophytes*; Stadler and Uber (1942) for maize pollen grains; Hollaender . . . Demerec (1945) for *Neurospora*; Hollaender and Zimmer (1945) for *P. notatum*; and Hollaender, Raper, and Coghill (1945) for *A. terreus*. The similarity between the action spectrum for mutation production and the absorption spectrum for nucleic acid is commonly interpreted to indicate that the nucleic acid portion of the gene acts as the chromophore for mutation production. Changes in viscosity, stream birefringence, and colloid osmotic pressure of deoxyribonucleate following ultraviolet irradiation have been studied by Hollaender *et al.* (1941). The doses required to produce a detectable effect *in vitro* are large compared to those required for the bactericidal or mutagenic effects.

Noethling and Stubbe (1934; Stubbe and Noethling, 1937) found 2967 Å to be the most efficient wave length of four tested in producing mutations when pollen of *Antirrhinum majus* was irradiated. Differential absorption of the irradiated pollen grains and the small number of tests cast doubt on the significance of this observation.

McAulay and his associates (McAulay and Ford, 1947; McAulay *et al.*, 1949; Ford and Kirwan, 1949) have published some unusual results obtained in studies of mutation induction with the fungus, *Chaetomium globosum*. The most efficient ultraviolet wave length is 2804 Å, and mutations are reported following irradiation with wave lengths of 3354, 3654, and 4047 Å. They report that the shorter wave lengths, i.e., 2654, 2804, and 2967 Å, selectively induce the so-called "K mutant," whereas very few K mutants are found following the near-ultraviolet and short-visible treatment. The statistical validity of this conclusion seems questionable since the sizes of samples observed are quite small. No K mutants were observed following X irradiation, whereas, with large doses of 2804 Å ultraviolet, as high as 62 per cent of K mutants were observed. The mutation-dose curves for "lethal mutations" (mutations resulting in cessation of growth while the colony is still very small) indicate that, although two quantum hits of 2804 Å ultraviolet will induce the mutation, five quantum hits of X rays are necessary for lethality (Ford and Kirwan, 1949). These results are difficult to evaluate since, except for the lethal-mutation studies, sample sizes are very small owing to the characteristics of the fungus which produces huge colonies. That the lethal mutations



are actually gene mutations seems doubtful since up to 100 per cent of such mutations can be induced at high doses.

Kaplan (1952) was the first to publish an action spectrum for mutation induction in bacteria. He studied the mutagenic efficiency of five wave lengths ranging from 2480 to 3030 Å. A maximum at 2650 Å was observed with each of the three types of mutations in *Serratia marcescens* studied. Similar results have been obtained by Zelle and Hollaender (unpublished data), who used six wave lengths from 2378 to 2967 Å. Maximum efficiency of mutation induction at 2650 Å was observed for both the streptomycin dependence to independence and the purineless reverse mutations in *E. coli* B/r. These are the same mutation systems that were employed by Anderson (1951b) in his study of the relation of oxygen concentration to mutation production by X rays. Absolute increases in the number of mutant cells were observed by Kaplan and by Zelle and Hollaender, thus eliminating differential survival of spontaneous mutants as the reason for the increased mutation rate. The observation of a maximum efficiency at 2650 Å in bacteria, as in sexually reproducing forms, is additional supporting evidence of the genic nature of bacterial variation.

#### INDUCTION OF MUTATIONS BY IRRADIATED MEDIA

In many of the earlier studies of bactericidal effects, the bacteria were irradiated on the surface of agar plates. To test the possibility of an indirect action through the photochemical production of toxic substances in the medium, several workers irradiated media before inoculation. In general, it was possible to demonstrate toxic properties of the irradiated media, but the doses required to produce an appreciable degree of toxicity were far beyond those utilized in the bactericidal tests by direct irradiation of the organism (Loofbourow, 1948).

The discovery of photoreactivation and of the influence of oxygen concentration on the bactericidal and mutagenic effects of ultraviolet and X rays, respectively, indicate clearly that at least part of the bactericidal and mutagenic effects of radiation are indirect and involve largely unknown reactions which, initiated by the radiation, ultimately alter a vital structure of the cell and cause either mutation or inactivation.

Interest in the indirect effects of the irradiation of substrates has been revived by a series of publications by a group at the University of Texas. Mutations to resistance to antibiotics in *S. aureus* were usually studied. Thus Stone *et al.* (1947) demonstrated that irradiation of the substrate with the entire spectrum of a quartz mercury arc lamp, prior to inoculation with bacteria, caused an increase in the mutation rate. Chemical treatment of the substrate or of amino acids added to the substrate with hydrogen peroxide caused a similar increase in mutations (Wyss *et al.*,

1947). Evidence was presented (Stone *et al.*, 1948) that selective growth of spontaneous mutations already present in the inoculum could not account for the observed results.

More critical evidence that irradiated substrate or peroxide-treated substrates could induce mutations was presented by Wagner *et al.* (1950) who observed increases in mutations in *Neurospora* conidia treated with irradiated broth. An increase in mutation rate when inhibitors of catalase and the cytochrome system were incorporated into the medium, thus permitting an accumulation of hydrogen peroxide within the cell, was reported by Wyss *et al.* (1948) and Wagner *et al.* (1950).

Haas *et al.* (1950) summarize these results and, in addition, report that the most effective wave lengths in producing mutagenic substrates are those below 2000 Å. Increasing the temperature of the substrate during the preinoculation irradiation from 0° to 60°C results in a significant increase in the mutagenic properties. They report that the bactericidal and mutagenic effects of irradiated medium were partially reversed by subsequent exposure to visible light.

Wyss *et al.* (1948) failed to demonstrate an increase in mutation rate by direct treatment of bacteria with hydrogen peroxide, although positive results were obtained by Wagner *et al.* (1950) with *Neurospora*. They conclude that formation of organic peroxides is responsible for the mutagenic effects of peroxide treatment of the broth and probably also of ultraviolet irradiation. This conclusion is strengthened by the work of Dickey *et al.* (1949), who showed that various organic peroxides are effective in inducing reverse mutation in an adenine-requiring strain of *Neurospora*. In their experiments the efficiency of the mutagenic peroxides was considerably less than that of direct ultraviolet irradiation.

Summarizing, it seems established that the mutation rate is increased when the cells are grown in ultraviolet-irradiated or hydrogen peroxide-treated substrates. It is difficult, however, to assess properly the significance of these results as they relate to the induction of mutations by direct irradiation of the cells with ultraviolet. In general, the mutagenic efficiency of the various substrate treatments is low; where comparisons were made, it is lower than that of direct ultraviolet radiation. Furthermore, there is a complete lack of quantitative data as to the amounts of radiation absorbed by the substrates. It is certain, however, that doses far greater than those employed in direct irradiation of cells are employed in the substrate treatments. Furthermore, the wave lengths effective in producing mutagenic compounds in irradiated substrates lie mainly below 2000 Å and are not present in the spectrum of the widely used germicidal lamps with glass envelopes or in monochromatic beams of ultraviolet radiation of the wave lengths most efficient in producing mutations by direct irradiation. Loofbrourow (1948) points out that, although the formation of hydrogen peroxide by ultraviolet radiation is essentially con-



finer to wave lengths shorter than 2000 Å, the photodecomposition of peroxide occurs throughout the ultraviolet spectrum.

All these considerations plus the selective absorption of ultraviolet by certain protoplasmic constituents indicate that only a very minor portion of the effects of direct ultraviolet irradiation of bacterial cells by the wave lengths usually employed can be ascribed to an indirect mechanism which has as its first step the formation of hydrogen peroxide from water.

A more conservative estimate of the significance of these results is that treatment of nutrient broth or solutions of certain amino acids with hydrogen peroxide or with ultraviolet of wave lengths shorter than 2000 Å can produce chemical mutagens. Such irradiation of so complex a substrate as nutrient broth would be followed by a variety of chemical changes including formation of hydrogen peroxide and organic peroxides. Since both hydrogen peroxide and certain organic peroxides have been shown to be mutagenic (Dickey *et al.*, 1949; Wagner *et al.*, 1950; Demerec, Bertani, and Flint, 1951), it is not surprising that broth irradiated under such conditions is also mutagenic.

#### MECHANISM OF RADIATION EFFECTS

Various hypotheses have been proposed to explain the bactericidal effects of radiations. In view of the recent evidence that a large proportion of the effects of both ionizing and ultraviolet radiation are produced by indirect mechanisms, many of the discussions are no longer appropriate, and no attempt will be made to reconsider them completely.

Such discussions have generally been concerned with two central problems: (1) how best to account for the kinetics observed, and (2) the nature of the damage and the mechanism by which it is produced.

In regard to the former problem, several writers (e.g., Gates, 1929a) have explained the occurrence of the survival curves observed on the basis of variation in resistance of the individual cells comprising the bacterial population. Although the occurrence of the sigmoidal survival curves can be explained on this basis with not too violently skewed distributions, the distribution required to explain an exponential survival curve, namely, an exponential distribution of resistances, is implausible. By contrast, the first-order kinetics indicated by exponential survival curves are a natural consequence of the target theory in its simplest form. Sigmoidal survival curves are easily accounted for on the basis of the multihit theory. Consequently, very few investigators still hold to the distribution theory.

In regard to the latter problem, a number of explanations have been proposed which are discussed by Lea *et al.* (1936). These may be broadly grouped into two categories: (1) secondary poisoning of the cell by chemical substances produced by the radiation and (2) decomposition of molecules vital to the organism. Lea and coworkers reject the poison

hypothesis on several grounds. It is difficult to account for the kinetics observed without making assumptions which are somewhat contradictory to the behavior of chemical disinfectants in bacteria. Furthermore, the independence of bactericidal effect and intensity and the lack of a large temperature coefficient characteristic of chemical disinfectants are likewise difficult to explain unless it is assumed that a cell poison produced internally by radiation behaves quite differently from a chemical disinfectant applied externally.

Postulating that a cell poison is produced by radiation really does little to explain the mechanism of radiation damage except to infer that an indirect mechanism exists between the initial chemical change produced by the radiation and the lethal end result. To be meaningful, the hypothesis must be elaborated to indicate the nature of the lethal effect produced and to take into account the kinetics observed. This has been done by Roberts and Aldous (1949) to explain their results on recovery of *E. coli* B from the effects of ultraviolet irradiation. They postulate that a cell poison is produced by the radiation by photochemical reaction and that this cell poison selectively affects the division mechanism. To explain the kinetics involved in the recovery, they further postulate that one molecule of this poison is effective in inhibiting division and, furthermore, that the poison exponentially disappears independently of ultraviolet irradiation.

Similarly, although a target-theory interpretation can adequately account for the kinetics observed, it has little meaning unless it is expanded to indicate the nature of the target and the change induced in the target by the radiation. Lea and his associates (1941; Lea, 1947) have done this and postulate the nucleus as the target and the induction of lethal mutations as the damage causing inactivation. On this hypothesis, Lea has estimated that *E. coli* possesses 250 genes capable of lethal mutation, each 12 m $\mu$  in diameter.

In view of the developments since 1947, however, a reexamination of the lethal-mutation interpretation seems indicated.

The growing body of evidence indicates that a major proportion of the effects of both ultraviolet and ionizing radiation on bacteria are indirect and involve a largely unknown chain of reactions occurring between the initial ionization, or quantum absorption, and the final lethal or mutagenic change. The assumption that the residual effect of radiations, i.e., the nonphotoreactivable portion of ultraviolet effects or the residual bactericidal effects of ionizing radiations in the absence of oxygen, is due to direct effects of the radiation on vital cell structures is by no means proved; the "residual effect" may actually involve still other indirect mechanisms.

The existence of an indirect mechanism, however, does not necessitate abandoning the assumption that the final decisive end product of the



chain of reactions is the induction of lethal or viable mutations. Indeed, the evidence indicates that with ultraviolet radiation the same photo-reactivable process affects both the bactericidal and mutagenic effects of radiation. Similarly, Anderson (1951b) has shown that in the case of one mutation system the lethal and mutagenic effects of X rays may result from the same indirect mechanism. Thus, although the existence of indirect mechanisms necessitates a modification of the concept of lethality by chemical change in one molecule, presumably the gene, by direct ionization, it does not require abandonment of the hypothesis that the final product of the indirect process may be lethal mutations. The observation of exponential survival curves when *E. coli* are X-irradiated in oxygen-saturated media indicates that first-order kinetics result, even though it is known that a large proportion of the effects are indirect. Thus the problem of determination of the best means to account for the observed first-order kinetics still remains, and the plausibility of the lethal-mutation theory is in no way impaired.

There are a number of observations, however, which bear directly on the lethal-mutation hypothesis.

Lea *et al.* (1937) and Witkin (1947) have observed that the long filamentous forms which result from bacteria subjected to sublethal doses of radiation react to subsequent exposure to radiation in the same manner as normal cells and appear to be equally sensitive to radiation. Delaporte (1949) has studied the filamentous forms of *E. coli* B cytologically and reports them to be multinucleate. It would thus appear that the mechanism of killing of such an elongated, multinucleate cell would not be due to recessive lethal mutations, and, if the lethal-mutation hypothesis is to be retained, it must be postulated that the induced lethal mutations are dominant and can be expressed equally well in uninucleate and multinucleate cells.

Demerec and Latarjet (1946) in comparative studies of *E. coli* B and B/r have shown that, with a given dose of ultraviolet, the rate of mutation induction is the same for the two strains, whereas the bactericidal effects are greatly different. A similar lack of correlation between the induction of mutations and the lethal effects was observed when comparisons were made with ultraviolet and X rays on strain B/r where, for a given survival ratio, the number of mutations induced by X rays was significantly less than the number induced by ultraviolet.

Beckhorn (1950) and Lederberg *et al.* (1951) have approached this question more directly by comparing the sensitivity of *E. coli* K12 cells, known genetically to be diploid, with that of haploid cells of the same strain. No systematic differences in sensitivity to ultraviolet radiation were observed, and the most prominent effect of irradiation appeared to be the conversion of diploid cells to the haploid condition. This haploidization occurs at doses smaller than those required for appreciable killing of

the cells. Beckhorn demonstrated that this unusual effect of ultraviolet was photoreactivable to about the same degree as are the bactericidal and mutagenic effects. Lederberg *et al.* have shown that X rays produce a similar effect and have found that, on continued incubation, most of the apparent haploid colonies develop spots of growth which are diploid. Thus, although diploid cells surviving the irradiation are temporarily altered so as to produce many haploid segregates, they retain the ability to transmit the diploid condition. These interesting results indicate that recessive lethal-mutation induction is not an important mechanism in killing by irradiation in these strains. If such were the case, marked differences in sensitivity should be observed between haploid and diploid cells and segregation of diploids to haploids should be decreased rather than stimulated since recessive lethals, although masked in the diploid, are expressed in the haploid cells.

That the nuclear constitution of microbial cells does influence the sensitivity to the lethal effects of radiation is indicated by the results of Latarjet and Ephrussi (1949) in which haploid and diploid cells of the same yeast strain were irradiated with X rays. They obtained exponential survival curves for the haploid cells and sigmoidal survival curves for the diploid cells. The sigmoidal curve corresponded approximately to a two-hit curve, as had been observed by other workers in studies of diploid yeast. Similar results have been obtained by Tobias (1952) who used haploid, diploid, and presumed tetraploid yeasts in tests of the diffusion model of the biological effects of high-energy radiations.

Atwood (1952) has devised a technique employing heterokaryotic conidia of *Neurospora* which permits, in addition to determination of the surviving fraction of cells, the determination of the surviving fraction of nuclei, the fraction of nuclei containing at least one recessive lethal mutation, the frequency with which separately induced recessive lethal mutations are homologous, and the degree to which all these effects are independent of one another. Although these studies are still in the preliminary stage, it seems warranted to conclude that killing of *Neurospora* conidia by X rays is primarily, if not entirely, a nuclear phenomenon and that recessive lethal mutations, although more important in uni-nucleate conidia, have but a minor effect in the inactivation of heterokaryotic nuclei since homologous recessive lethal mutations must be induced for their expression. Utilizing similar techniques, Norman (1951) has published extensive experiments on the ultraviolet inactivation of *Neurospora* conidia. He concludes that inactivation of conidia is a consequence of the inactivation of nuclei and that nuclei exhibit first-order kinetics. Two kinds of inactivation processes are postulated: recessive lethal mutations and a nongenetic effect on the nucleus. The two mechanisms are intimately related, however, since the same action spectrum is obtained with monochromatic ultraviolet radiation and each



is photoreactivated to about the same extent. No speculations concerning the nature of the nongenetic nuclear damage are given, but it would seem that it may be of a gross nature, and hence may involve a number of genes. The observation of first-order kinetics indicates that the ultraviolet quantum is capable of producing this effect.

These observations with yeast, and especially with *Neurospora*, indicate that the damage causing lethality is damage to the nucleus, i.e., to the genetic apparatus. Furthermore, the *Neurospora* results indicate that recessive lethal mutation is of relatively minor importance in the inactivation of multinucleate cells by irradiation. Since there is an increasing amount of evidence that bacterial cells of certain species may be multinucleate, at least at certain stages of development of the culture, a similar situation may obtain in bacteria. This has been indicated by the results of Stapleton (1952), who found differences in X-ray resistance and in the kinetics of inactivation which were correlated with differences in the mean number of nuclei of *E. coli* B/r cells at different stages of the growth cycle.

Witkin (1951), however, found resistance of *E. coli* B/r to ultraviolet to be lowest in growing cells, greater in resting cells, and highest in cells in the lag phase. These differences in resistance did not parallel differences in the average number of nuclei—4, 2, and 4, respectively—for the three stages of the growth cycle and seem incompatible with the recessive lethal-mutation hypothesis of radiation killing. Furthermore, the correlation between nuclear number and the size of lactose negative sectors in colonies derived from cells which survive irradiation strongly suggests that nuclear segregation is partially responsible for the sectoring colonies. If this is actually the case, it is questionable if ultraviolet killing is nuclear at all since, as Witkin points out, with survivals as low as  $10^{-3}$  as in her experiments, the probability of a surviving cell having more than one viable nucleus would be very low.

Dale (1940, 1942) has suggested that the bactericidal effects of irradiation may not involve lethal mutations but rather the inactivation of enzymes. Although objections to this hypothesis have been made on the basis of the relative insensitivity of enzymes irradiated *in vitro*, Dale has shown that dilute solutions of purified enzymes are quite sensitive to radiation, and, indeed, this sensitivity is the basis for his suggestion that enzyme inactivation may be a factor in the bactericidal effects of irradiation. Barron *et al.* (1949) have shown that the sulfhydryl-containing enzymes are particularly sensitive to ionizing radiations. It is known that dilute solutions of enzymes are inactivated primarily by an indirect effect of ionizing radiations and that a great variety of compounds protect the enzyme against radiation by competing for the highly reactive products formed from water. It would appear that a similar competitive protection would occur within the cell, since a great variety of proteins are

present. A further difficulty arises in accounting for the first-order kinetics frequently observed. If there are many molecules of a given enzyme present in the cell, it is difficult to visualize that destruction of a single such molecule would be lethal to the cell. McIlwain (1946) has suggested that only one or a very few molecules of certain enzymes may be present in the cell. If this is the case, the first-order kinetics can be explained. If the extreme possibility, suggested by McIlwain (1947), that the one or few units of enzyme present in the cell are actually the units of inheritance, is correct, the lethal-mutation and enzyme-destruction hypotheses of the ultimate damage responsible for the bactericidal effects merge.

There is increasing evidence to indicate that attempts to explain the bactericidal effects of irradiation on the basis of one mechanism are not realistic. Luria (1939), on the basis of microscopic examination of incubated irradiated cells, suggested that more than one mechanism of killing existed and that an attempt to explain the bactericidal effects of irradiation on the basis of a single mechanism did not seem warranted. For both X rays and ultraviolet, an indirect mechanism is known to produce a portion of bactericidal effects, whereas apparently quite different mechanisms account for the residual effects, although the ultimate damage may be the same in both cases. It is interesting to compare some of the known facts concerning *E. coli* B and B/r from the standpoint of multiple mechanisms.

Apparently, these two strains differ by mutation of a single gene. Even though closely related, they show many differences in behavior following ultraviolet irradiation. Strain B is much more sensitive than the usual *E. coli* strain (Tables 10-2 and 4), and Witkin (1947) and Roberts and Aldous (1949) have shown that a large part of the killing seems to involve damage to the cell-division mechanism. *E. coli* B exhibits exponential killing with ultraviolet, is one of the few bacterial strains found to exhibit heat reactivation (Anderson, 1949, 1951a; Stein and Meutzner, 1950), recovers partially when held in liquid media (Roberts and Aldous, 1949), and does not exhibit catalase reactivation (Latarjet and Caldas, 1952). Strain B/r is much more resistant both to ultraviolet and X rays than strain B, exhibits a sigmoidal survival curve with ultraviolet, shows only a small degree of heat reactivation, does not recover from ultraviolet irradiation in liquid suspension (Roberts and Aldous, 1949), and exhibits a small but definite amount of catalase restoration. Both strains exhibit photoreactivation, and Demerec and Latarjet (1946) have shown that, for equal doses of ultraviolet, the rate of phage resistance-mutation induction is the same in each strain.

At least two different mechanisms must be postulated to explain these differences in behavior of the two strains following ultraviolet irradiation, and the catalase restoration may necessitate a third. Since both B and B/r exhibit photoreactivation, it seems likely that the initial photo-



chemical events may be similar in the two organisms, and involve the production of a product sensitive to visible light which subsequently initiates in the two organisms two different series of reactions leading to different, ultimately lethal effects. It is possible that both mechanisms are operative in both strains with the difference in sensitivity due to the single gene mutation being a shift in the relative proportion of effects of radiation produced by the two mechanisms. With both B and B/r a non-photoreactivable residue exists, making it seem necessary to postulate in each strain an additional mechanism which is at least partially distinct from the photoreactivable mechanism.

To these mechanisms involving ultraviolet must be added still different mechanisms for X rays since no photoreactivation occurs and, in strain B, no filamentous forms are produced (Roberts and Aldous, 1949). The X-ray effects, in turn, are subdivided into an oxygen-influenced and an oxygen-independent mechanism. As with the photoreactivable and non-photoreactivable mechanisms of ultraviolet, it is not clear if the oxygen-dependent and oxygen-independent effects of X rays produce the same or different kinds of ultimately lethal damage to the cells. Thus the observations on these two closely related strains indicate that a variety of mechanisms exist by which radiations produce bactericidal effects.

In view of the ionization-density studies, it seems likely that different mechanisms are involved in the inactivation by high-energy radiations of spores and vegetative cells.

A quite different mechanism by which radiation can cause the killing of bacterial cells has been demonstrated by Lwoff *et al.* (1950) in studies of lysogenic strains of bacteria. It was found that small doses of ultraviolet will induce, in nearly all the cells, the formation of active bacteriophage resulting in lysis. Latarjet (1951) has shown that small doses of X rays have a similar effect and, furthermore, that visible light reduces the effect following both ultraviolet and X irradiation. Similar ultraviolet treatment will cause phage production and lysis of *E. coli* K12 (Weigle and Delbrück, 1951) which E. Lederberg (personal communication) discovered to be lysogenic.

A similar variety of mechanisms may be involved in mutation production since it is known that mutations are induced by the photoreactivable and nonphotoreactivable ultraviolet mechanisms and by X rays both in the presence and in the absence of oxygen. In addition, it is becoming apparent that different loci may respond quite differently to a particular radiation, as evidenced by the different types of mutation-dose curves and the differential response of two different mutations to X rays in the presence and absence of oxygen.

Many problems have been uncovered which, when more thoroughly analyzed, promise to add much to the knowledge of the effects of irradiation in bacteria. In the more detailed analysis of the problems, parallel

studies of the genetic and lethal effects seem desirable, whenever possible, in order to determine the extent to which the mechanisms involved in producing these effects are common or distinct.

Some of the problems which seem worthy of more detailed study are photoreactivation, the indirect and presumed direct effects of X rays, the various types of recovery which have been observed, the differences in response of cells grown under different conditions, and chemical protection. Additional investigations of the physiological properties of irradiated cells may help to determine the nature of the ultimate lethal damage produced by irradiation. Even the kinetic picture is not clear, and studies of inactivation (e.g., Stapleton, 1952) and parallel studies of killing and mutation (e.g., Witkin, 1951), both of which take into account the nuclear constitution of the cells, seem especially valuable.

Although much has been learned, especially during the past decade, concerning radiation effects on bacteria and the mechanisms whereby they are produced, the advances in the immediate future seem likely to far surpass those of the past, for there have never been so many promising approaches from different directions utilizing such critical techniques as are now available.

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## CHAPTER 11

### Radiation Studies on Fungi<sup>1</sup>

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*Introduction: Ultraviolet radiation: Wave-length dependence in the ultraviolet—Survival and mutation kinetics—Modifying effects on ultraviolet action. Ionizing radiations: Comparison of different ionizing radiations—Dose-effect curves—Modifying factors—Conclusions. References.*

#### INTRODUCTION

The desirability of fungi as objects of radiobiological investigation arises simply from the ease of experimentation with the various kinds of durable, easily enumerable spores or other formed elements which are available. Certain fungi offer the combined advantages of the mass techniques peculiar to microbial genetics and the opportunity for detailed genetic analysis. As might be expected, no general lines of approach have been developed exclusively in the fungi, and the experimental work cannot be said to form a unified body of fact. It is the purpose of the present review to indicate briefly the sources and significance of divers contributions in the area of radiation effects in this group of organisms. No particular effort has been made to cover work done prior to 1936, when a review of this subject was presented (Smith, 1936).

Numerous agents are well known for their ability to cause genetic changes in biological material, and many, if not all, have been used on fungi. Of prime importance for this discussion are, of course, various radiations. These include ultraviolet, X rays,  $\alpha$  particles, and neutrons. By far the greatest amount of work on fungi has been done with X and ultraviolet radiations. Various chemicals are also known to be capable of causing changes similar to those caused by radiations. From the standpoint of radiation biology, the study of radiomimetic chemicals is interesting because of the possibility that a large portion of the effects of

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radiation may proceed through the action of chemical intermediates. Moreover, it may be possible to deduce important characteristics of the radiosensitive cell constituents from a knowledge of the reactions in which radiomimetic chemicals will specifically participate. Discussions of chemical mutagenesis dealing mainly with *Neurospora* have appeared (Tatum, 1950; Jensen, *et al.*, 1951). Table 11-1 is representative but is by no means an exhaustive list of chemicals for which claims of mutagenic activity have been put forth.

TABLE 11-1. SOME CHEMICALS PRODUCING RADIATIONLIKE EFFECTS IN FUNGI

Chemical	Fungus	Reference
Mustard gas.....	<i>Neurospora crassa</i>	Horowitz <i>et al.</i> , 1946
Mustard gas.....	<i>Aspergillus nidulans</i>	Hockenhull, 1948
Nitrogen mustard.....	<i>Neurospora crassa</i>	McElroy <i>et al.</i> , 1947; Miller and McElroy, 1947, 1948
Nitrogen mustard.....	<i>Coprinus fimetarius</i>	Fries, 1948
Nitrogen mustard.....	<i>Penicillium notatum</i>	Stahmann and Stauffer, 1947
Nitrogen mustard.....	<i>Saccharomyces cerevisiae</i>	Reaume and Tatum, 1949
20-Methylcholanthrene-endosuccinic acid....	<i>Neurospora crassa</i>	Tatum, 1947
Camphor.....	<i>Saccharomyces</i> sp.	Subramaniam and Rao, 1950, Skovsted, 1949, 1948
Acenaphthene.....	<i>Saccharomyces</i> sp.	Subramaniam and Murthy, 1949
Sodium nitrite Ninhydrin Chloramine T Potassium iodide	<i>Aspergillus</i> sp.	Steinberg and Thom, 1940, 1942
Organic peroxides.....		
Hydrogen peroxide and potassium cyanide...		
Caffeine.....		
Methyl xanthines.....	<i>Ophiostoma multiannulatum</i>	Fries, 1950
Diazomethane.....	<i>Ophiostoma multiannulatum</i>	Fries and Kihlman, 1948
Acriflavine.....	<i>Neurospora crassa</i>	Jensen <i>et al.</i> , 1949
	<i>Saccharomyces cerevisiae</i>	Ephrussi and Hottinguer, 1950

It should be noted, particularly for chemical mutagens, that the mutations observed may not always be caused directly by the agents used or in the manner anticipated. The unmasking of genetic variability, which is concealed in polyploid or particularly in heterokaryotic complexes, is a basis for induced variation in fungi which, if unrecognized, may lead to erroneous conclusions about the actions of inducing agents. It is also important to examine the experimental conditions to evaluate the possibility of selection of preexisting spontaneous mutants as opposed to direct causation (Lederberg, 1948), an evaluation which has not always been critically attempted. Spontaneous genetic changes are well known both

in natural and laboratory cultures and many of the induced changes resemble those already seen in nature, a fact appreciated very early by investigators of fungi (e.g., Nadson and Philippov, 1932). This reflects the nonspecific character of the action of radiation and radiomimetic chemicals which typically increase the incidence of many different kinds of mutations simultaneously.

It is reasonable to suppose that there must be many separate effects of radiation which pass unnoticed because the means for detecting them are lacking. Some of these may be biologically unimportant, whereas others may provide the underlying basis for some of the major observed results. For example, the number and character of the lethal effects are not known, although there is reason to suspect that many alternatives are involved. It will often be difficult to find adequate criteria to distinguish between primary effects of radiation and the secondary consequences of cell morbidity, a ubiquitous problem in radiation biology.

### ULTRAVIOLET RADIATION

Several recent articles are available on general biophysical aspects of ultraviolet radiation, an agent which has been widely used in experiments with fungi (Loofbrourow, 1948; Giese, 1945, 1950; McLaren, 1949). Effects of ultraviolet on growth and respiration have been noted in yeasts and molds, as shown in Table 11-2.

TABLE 11-2. SOME NONGENETIC EFFECTS OF ULTRAVIOLET IRRADIATION OF FUNGI

Type of effect	Organism	Remarks	Reference
Stimulation.....	Various yeasts, and mucors	Low doses only	Nadson and Philip- pov, 1928a
Delayed budding or germination, retarded growth rate	Various yeasts: <i>Rhizopus suinus</i> <i>Ustilago zeae</i> <i>Aspergillus niger</i>	Low doses	Lacassagne, 1930; Dimond and Duggar, 1940a, b; Landen, 1939; Zahl <i>et al.</i> , 1939
Changes in respiration	<i>Saccharomyces</i> <i>cerevisiae</i>	.....	Giese, 1942; Giese and Swanson, 1947

Stimulation by slight doses has been reported with various yeasts (*Saccharomyces cerevisiae*, *Saccharomyces ellipsoideus*, *Nadsonia fulvescens*, *Zygosaccharomyces priorianus*) and molds (*Mucor genevensis*, *Mucor guilliermondi*), based on increased growth on plates exposed to a graded amount of radiation (Nadson and Philippov, 1928a). The interpretation of such experiments is made uncertain by the possibility that stimulation is brought about by substances released from the few killed cells.



Yeast cells irradiated with ultraviolet of wave lengths 2800–3800 Å (at somewhat higher doses than used by Nadson and Philippov, 1928a) did not begin to bud as early as the controls—in some cases they never budded, whereas in others they finally grew like the controls (Lacassagne, 1930). Other investigators, working with somewhat shorter wave lengths, also found delayed budding phenomena and killing in various species of *Saccharomyces* (Wyckoff and Luyet, 1931; Oster, 1934a, b, c). The spores of *Rhizopus suinus* show a similar behavior in that, after irradiation at 2650 Å, they exhibit delayed germination or complete lack thereof, as well as a somewhat retarded growth rate of the germinated spores (Dimond and Duggar, 1940a, b). *Ustilago zae* has been reported to behave in this way (Landen, 1939), as has *Aspergillus niger* (Zahl *et al.*, 1939).

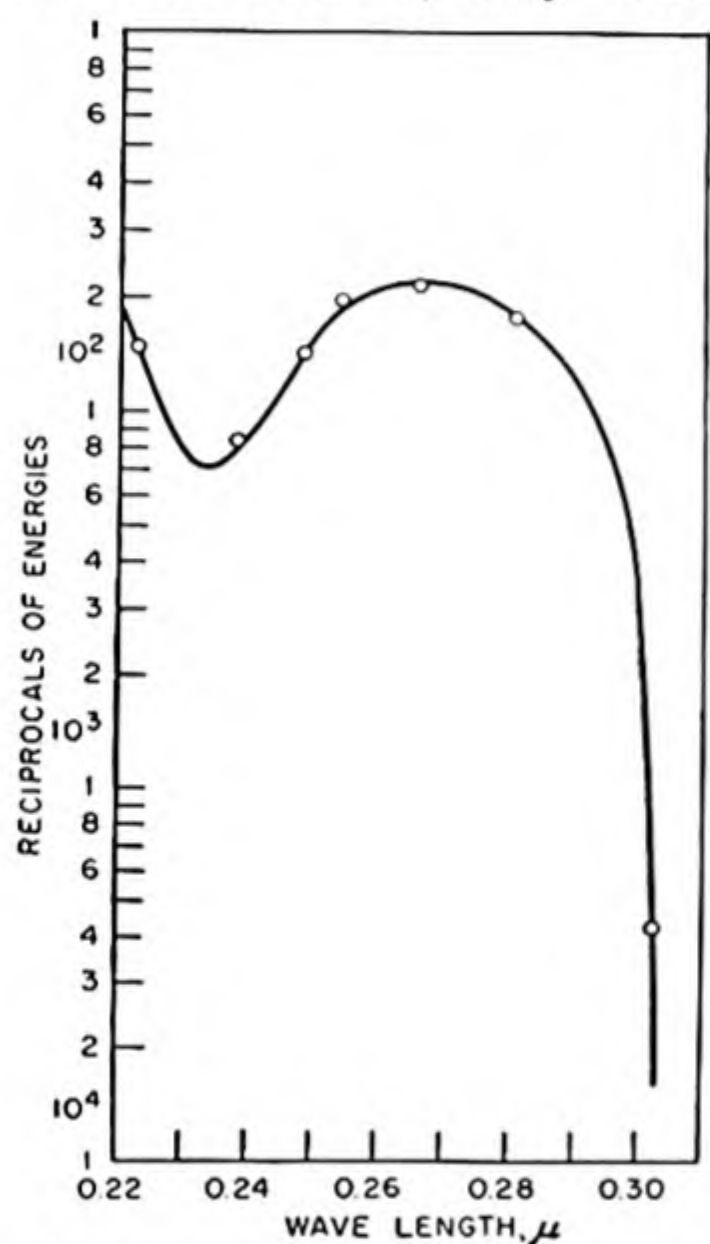


FIG. 11-1. Comparison of the destructive efficiency of ultraviolet energy on yeast at different wave lengths. Ordinate, reciprocals of the energies required to "kill" 50 per cent of the yeast cells. (Oster, 1934c.)

effects of ultraviolet irradiation may result from the absorption of energy by . . . nucleoproteins" (Oster, 1934c), because of the correspondence between the killing action spectrum and the absorption spectrum of nucleoproteins (see Loofbourow, 1948; Giese, 1950, for discussions of the significance of action spectra). Similar results for killing and mutation have been obtained in various fungi as shown in Table 11-3. In a series of papers dealing with *Chaetomium globosum* a sectorial colonial change, designated as "K," has been found to be selectively produced at

thereof, as well as a somewhat retarded growth rate of the germinated spores (Dimond and Duggar, 1940a, b). *Ustilago zae* has been reported to behave in this way (Landen, 1939), as has *Aspergillus niger* (Zahl *et al.*, 1939).

Effects on respiration have been observed by various workers (Oster, 1934a; Giese, 1942; Giese and Swanson, 1947). There appears to be an increase in endogenous respiration with a decrease in exogenous respiration of various substrates by *S. cerevisiae* (experimental conditions used by Giese, 1942, and Giese and Swanson, 1947:  $2 \times 10^8$  cells/ml in a quartz Warburg vessel, Sterilamp as source of ultraviolet radiation, 10- to 15-min exposures, approximate dose of 70 ergs/mm<sup>2</sup>/sec). A decreased fermentation rate was also observed (Giese and Swanson, 1947).

*Wave-length Dependence in the Ultraviolet.* For yeast, maximum killing efficiency was found to lie between 2600 and 2700 Å, and Fig. 11-1 (Oster, 1934c) is representative of the action spectra. The suggestion was made that "the

wave lengths around 2800 Å but not at longer wave lengths (around 3300 Å), while "saltations involving modifications of growth rate and form, mycelium and perithecia are produced" in constant proportion to lethal effect at all wave lengths tested (2300–4047 Å) (McAulay *et al.*, 1945; Ford, 1947). However, experiments of 7–10 days duration (as were the irradiation exposures at wave length 4047 Å) are open to question on the grounds of contamination by shorter wave lengths, as well as of changes in the organism. The percentage of colonies showing the K-type sector has reached as high in some experiments as 30–50 per cent (Ford, 1946), with the peak activity at 2804 Å (McAulay and Ford, 1947).

TABLE 11-3. ORGANISMS SHOWING WAVE-LENGTH DEPENDENCE FOR KILLING AND MUTATION WITH ULTRAVIOLET RADIATION

Organism	Remarks	Reference
<i>Saccharomyces cerevisiae</i> .....	2600–2700 Å max. kill	Oster, 1934c
<i>Ustilago zeae</i> .....	2400 Å max. activity; $1.5 \times 10^6$ ergs/mm <sup>2</sup> at 3130 Å produced no kill	Landen, 1939
<i>Aspergillus niger</i> .....	2537 Å max. activity; $2 \times 10^4$ ergs/mm <sup>2</sup> gave 30% kill at 3129 Å; no effect noted at 3650 Å	Zahl <i>et al.</i> , 1939
<i>Trichophyton mentagrophytes</i>	2650 Å max. activity; kill but no mutation between 3400 and 4400 Å	Hollaender and Emmons, 1939, 1941, 1946; Emmons and Hollaender, 1939
<i>Aspergillus terreus</i> .....	2537–2650 Å max. activity; 2967 and 3150 Å produce mutation but less efficiently	Hollaender and Emmons, 1946; Hollaender, Sansome, <i>et al.</i> , 1945
<i>Neurospora crassa</i> .....	2650 Å max. activity; 2280 Å gave kill but little mutation; at 2967 Å both were low	Hollaender, Sansome, <i>et al.</i> , 1945
<i>Penicillium notatum</i> .....	2650 Å max. activity	Hollaender and Zimmer, 1945
<i>Chaetomium globosum</i> .....	Exception to general observation; see text for discussion	McAulay <i>et al.</i> , 1945; Ford, 1946, 1947; McAulay and Ford, 1947

The wave-length dependence of the production of K type and other saltants led to two conclusions: (1) a protein excitation near 2800 Å gives rise to K type, and is responsible for most efficient production of other mutants, and (2) a second, less efficient process, can occur at all wave lengths tested to produce mutants other than K type (McAulay and Ford, 1947). Figures 11-2a,b graphically summarize these points. The major emphasis was that *protein* absorption, not nucleic acid absorption, gave



rise to K mutations in this material. X rays were found not to produce the K saltation (Ford and Kirwan, 1949; Ford, 1948; McAulay *et al.*, 1949), but other morphological changes were produced. So-called "lethal mutations" were found with all wave lengths of ultraviolet examined and with X rays (Ford and Kirwan, 1949; Ford, 1948; McAulay *et al.*, 1949). These were, in fact, spores which germinated, produced a small amount of mycelium, and then stopped growing; it is impossible to say whether the cessation of growth is attributable to genetic mutation in the usual sense. Since *C. globosum* is apparently unsuitable for ordinary genetic experiments, the basis for the K saltation remains equally uncertain. In view

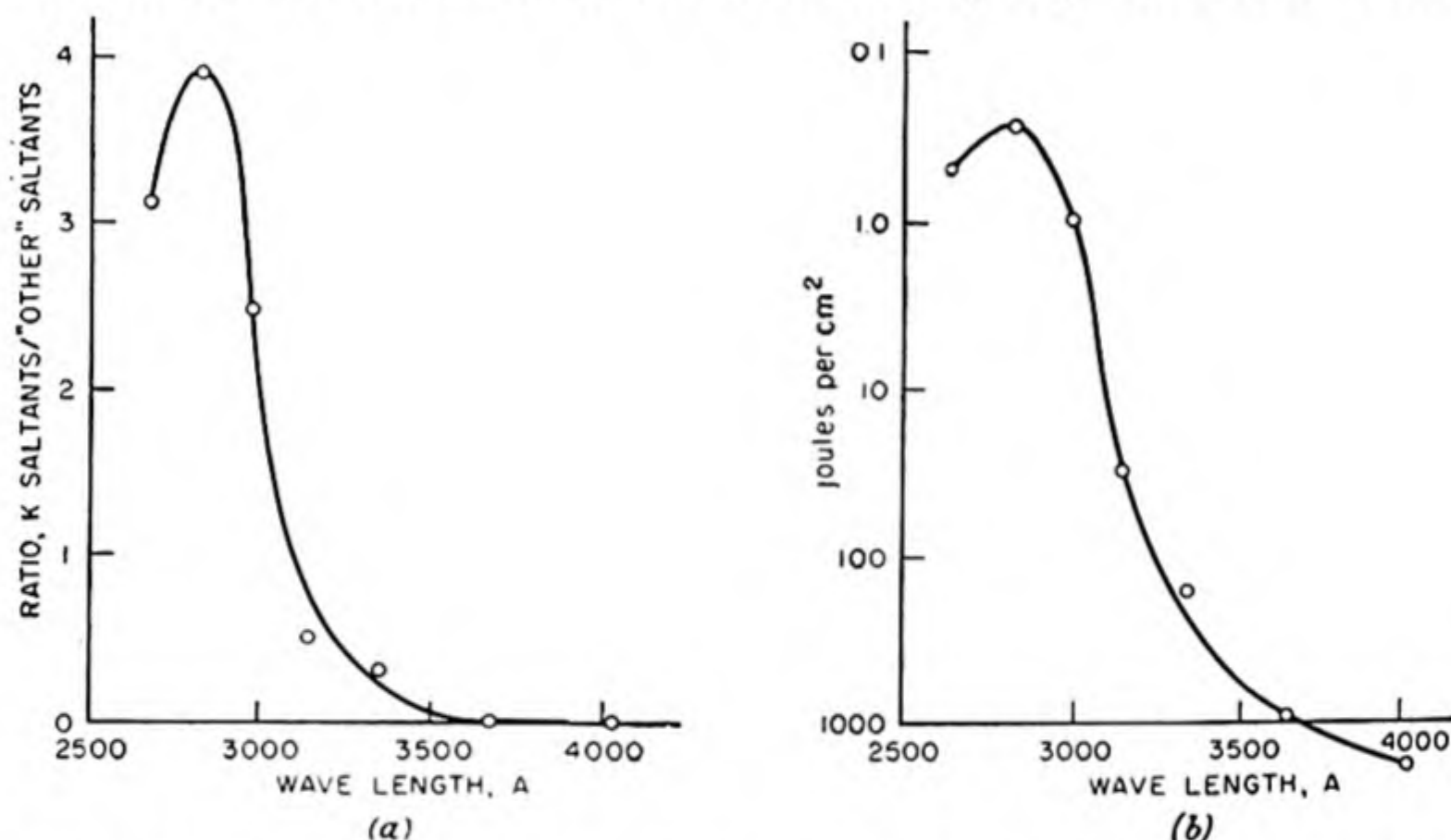


FIG. 11-2. Curve *a* shows the ratio of percentage K saltants to the percentage "other" saltants at different wave lengths. Curve *b* shows the optimum doses in joules per square centimeter for saltant production. Both curves are plotted against wave length: curve *a*, natural scale; curve *b*, logarithmic scale. Doses read downward to bring out the similarity between the curves. (McAulay and Ford, 1947.)

of these uncertainties and of the general finding in other material of action spectra suggesting nucleic acid rather than protein excitation it seems reasonable to suppose that the interesting findings in *Chaetomium* require an ad hoc explanation, and are not to be regarded as serious obstacles to the hypothesis that nucleic acid excitation is the most effective primary action of ultraviolet.

It should be noted that the finding of a marked effect (in terms of killing and mutation) of ultraviolet-irradiated medium (Wagner *et al.*, 1950) raises some question as to the complete propriety of any interpretation based solely on a consideration of certain absorptive characteristics of the organism (Bacq, 1951). The experiments show that toxic and mutagenic substances are formed by the action of ultraviolet on nutrient broth. Similarly, treatment of nutrient broth with hydrogen peroxide produces mutagenic activity. There are important differences between

direct irradiation of the cells and irradiation of the medium, however. The former is so much more effective, dose for dose, that it seems hardly possible to interpret the difference solely in terms of greater absorption by the cells. Since activation of the medium is produced only by lamps with a quartz envelope, it appears that very short wave lengths, producing high yields of peroxide, may play an important role. Action spectra for mutagenesis by direct cell irradiation are clearly unrelated to the yield of peroxide. It is not yet possible to state to what extent the results produced by irradiating the cells directly are brought about by the same mechanisms which operate when the medium is irradiated in the absence of cells, but these mechanisms are most likely of secondary importance.

TABLE 11-4. TYPES OF SURVIVAL CURVES OBTAINED AFTER ULTRAVIOLET IRRADIATION

Organism	Type of curve	Morphological element irradiated	Reference
<i>Rhizopus suinus</i> } <i>Mucor dispersus</i> } . . . .	Sigmoidal	Conidiospores	Dimond and Duggar, 1941
<i>Aspergillus melleus</i> . . .	Exponential	Conidiospores	Dimond and Duggar, 1941
<i>Trichophyton mentagrophytes</i>	Sigmoidal	Conidiospores	Hollaender and Emmons, 1939, 1941, 1946; Emmons and Hollaender, 1939
<i>Aspergillus terreus</i> . . .	Sigmoidal	Conidiospores	Hollaender, Sansome, <i>et al.</i> , 1945
<i>Saccharomyces cerevisiae</i> (haploid)	Exponential	Resting cells	DeLong and Lindegren, 1951
<i>Saccharomyces cerevisiae</i> (haploid)	Sigmoidal	Resting cells	Sarachek and Lucke, 1953; Pomper, unpublished
<i>Saccharomyces cerevisiae</i> (diploid)	Sigmoidal	Resting cells	Wyckoff and Luyet, 1931; DeLong and Lindegren, 1951; Pomper, unpublished
<i>Ustilago zaeae</i> (haploid and diploid)	Sigmoidal	Sporidia and chlamydospores	Landen, 1939
<i>Streptomyces flaveolus</i>	Exponential	Conidiospores	Kelner, 1948
<i>Streptomyces griseus</i> . .	Sigmoidal	Conidiospores	Savage, 1949
<i>Aspergillus niger</i> . . . .	Sigmoidal	Conidiospores	Zahl <i>et al.</i> , 1939
<i>Rhizopus nigricans</i> . . .	Sigmoidal	Conidiospores	Luyet, 1932
<i>Neurospora crassa</i> . . .	Exponential	Uninucleate microconidia	Norman, 1951
<i>Neurospora crassa</i> . . .	Sigmoidal	Multinucleate conidia	Norman, 1951

**Survival and Mutation Kinetics.** There appears to be no clear separation of lethal and mutagenic effects on the basis of wave-length dependence, although nonmutagenic wave lengths can be lethal at very high doses. On the other hand, the dose-response curves for the two effects show marked differences. Table 11-4 lists the types of ultraviolet sur-



vival curves that have been reported with various fungi. On the basis of the analysis carried out with *Neurospora* (Norman, 1951), the occurrence of both sigmoidal and exponential survival curves among the fungi may reflect differences in the number of nuclei per spore. Exponential survival was obtained with uninucleate conidia of *Neurospora*, whereas sigmoidal survival was correlated with a multinucleate condition (Atwood

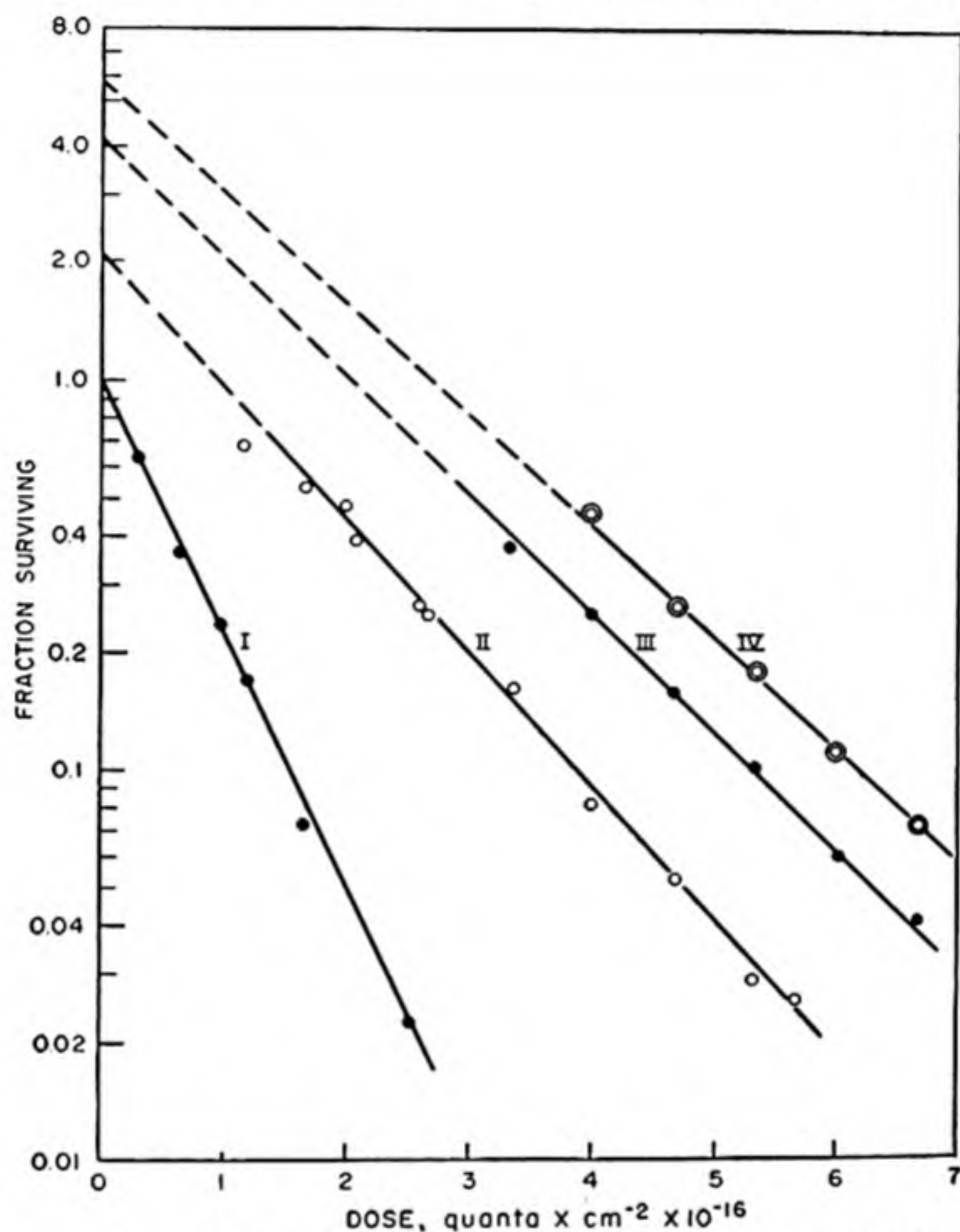


FIG. 11-3. Inactivation of *Neurospora* conidia by ultraviolet radiation. Uninucleate and multinucleate survival curves. Average number of nuclei per conidium: curve I, 1.0; curve II, 2.3; curve III, 4.2; curve IV, 5.9. (Norman, 1951.)

and Norman, 1949; Norman, 1951). Figure 11-3 shows the survival curves obtained by Norman with uni- and multinucleate conidia of *Neurospora*. Similarly, the ploidy has been implicated as an important factor in yeast. The ultraviolet survival curves in yeast are generally sigmoidal, but those of haploids are of much lower order than those of polyploids (Sarachek and Lucke, 1953; Caldas and Constantin, 1951; Warshaw, 1952; Pomper, unpublished).

Mutation frequency curves have been obtained with various fungi. *Trichophyton mentagrophytes* (Hollaender and Emmons, 1941), *Neurospora crassa* (Hollaender, Sansome, et al., 1945), *Aspergillus terreus*

(Hollaender, Raper, and Coghill, 1945), and *Penicillium notatum* (Hollaender and Zimmer, 1945) exhibit more or less similar behavior, in that mutation increases to a maximum and then decreases rather erratically (Fig. 11-4). Decrease in the mutation frequency at higher doses was not observed with *Streptomyces flaveolus* (Kelner, 1948), although rather erratic behavior was noted. A series of inositolless mutants of *N. crassa* have been used (Giles and Lederberg, 1948; Giles, 1948, 1951) to study the frequency of induced reversions to inositol independence. Similar experiments have been carried out with mutants of *S. cerevisiae* requiring adenine and uracil (Pomper, unpublished). Figure 11-5 shows a mutation frequency curve obtained with inositolless *Neurospora* (Giles, 1951). The mutation frequency in measurements of reversions of this sort is usually fairly low, so that accurate determinations cannot be made when the survivors fall below a certain level. On the other hand, the experiments described at the beginning of this paragraph measured occurrence of morphological mutations, which in some instances reached as high as 30-80 per cent. Hence, the curves could be followed over greater dose ranges using small cell populations. Also in these studies, many different "mutations" of uncertain genetic status were observed as compared to the more restricted assay when using reversions under conditions of controlled genetics. These differences may be part of the explanation for the difference in shape of the mutation curves. An anomalous relation between the ultraviolet dose-effect curves for survival and mutation production is particularly apparent in *Neurospora*. Although uninucleate microconidia of *Neurospora* give exponential survival curves, suggesting a single-hit or single-target event, they do not give linear mutation curves. It would seem logical to expect that the frequency of the mutational event should be simply proportional to dose, since deviations from such proportionality would, in the case of lethal mutation, produce corresponding deviations from the exponential survival curve. It has been

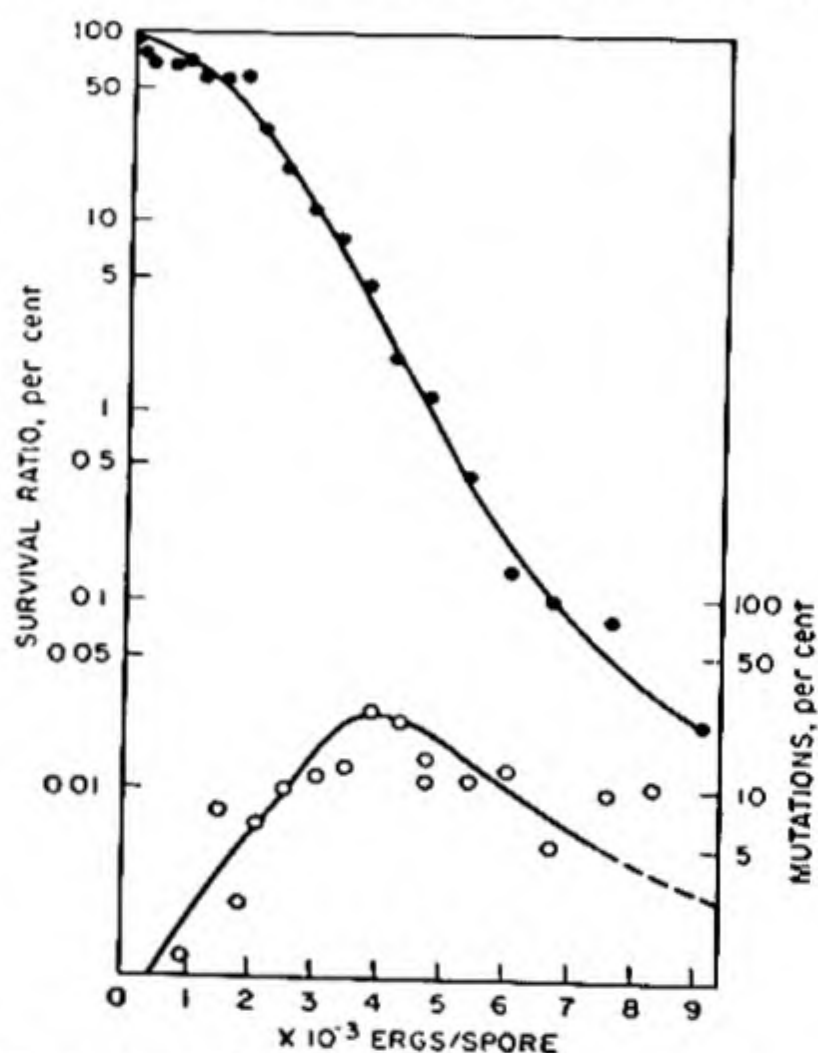


FIG. 11-4. Curve showing mutation production and killing. Top curve: change of survival ratio of fungus spores with increasing energy using 2650 Å radiation. Lower curve: variation of percentage mutations of surviving spores. Both curves were obtained from the same material. (Adapted from Hollaender and Emmons, 1941.)



shown by the heterokaryon method (Atwood, 1950) that sufficient recessive lethal mutations are induced in the nuclei of macroconidia to distort noticeably the exponential survival curve if these mutations were induced in micronidia according to the same dose-effect relation as in macroconidia. It is possible that an interaction of unknown nature between the nuclei of the multinucleate cells may be the explanation for this discrepancy.

Data on *C. globosum* have been interpreted as signifying a requirement of two quantum hits at 2804 Å to cause lethal mutation (Ford and Kirwan,

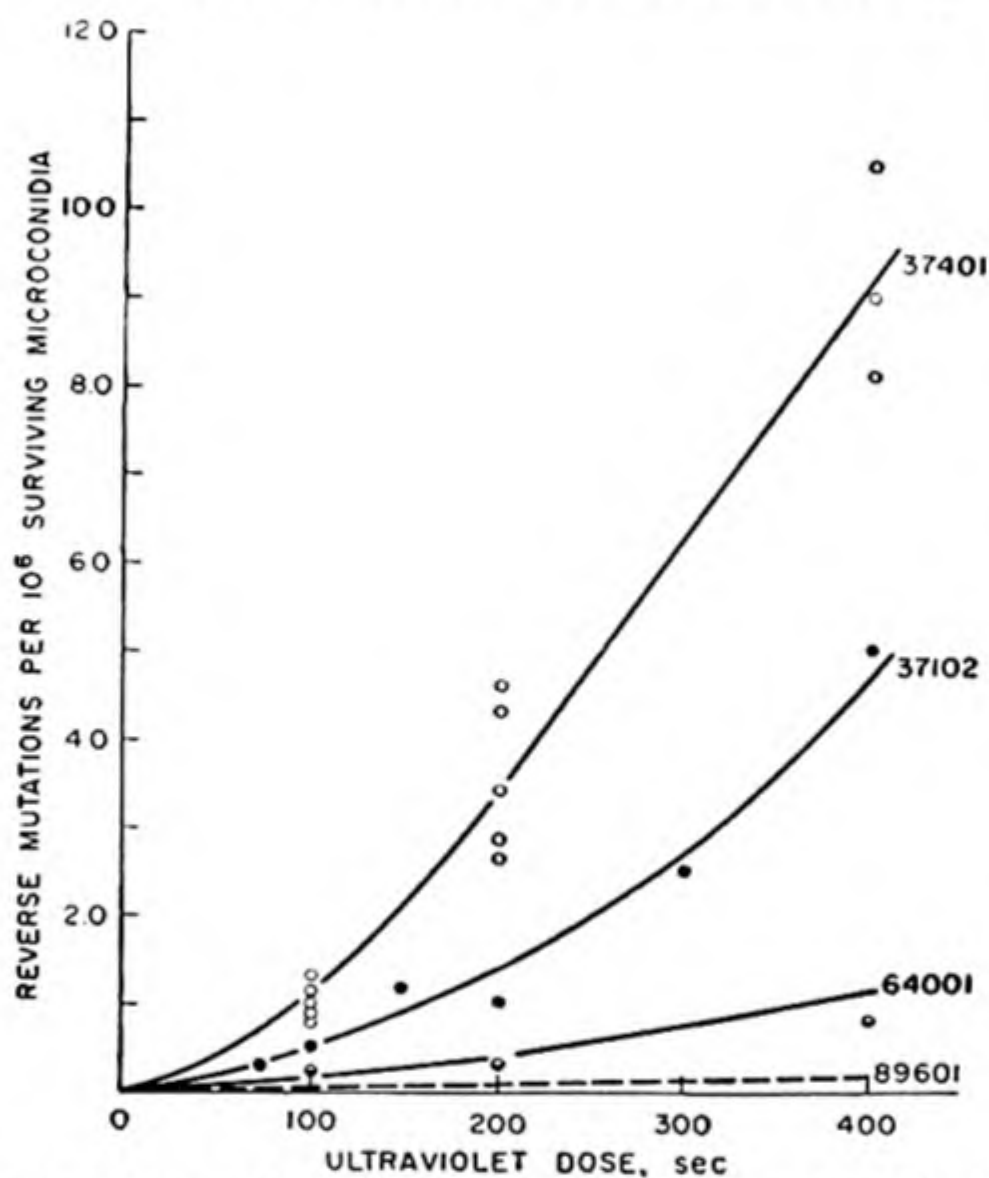


FIG. 11-5. Relation between ultraviolet dosage and frequency of reverse mutations in three inositolless mutants (all in *c m f* stocks). The dotted line for mutant 89601 is added to indicate the exceedingly low reverse-mutation rate for this mutant. (Giles, 1951.)

1949; McAulay *et al.*, 1949), and earlier work with yeast (Oster and Arnold, 1934) suggested that different numbers of hits are required to bring about different degrees of ultraviolet action, as judged by the criterion of the amount of budding prior to cessation of growth. Such observations may be indicative of a manifold inactivation unit allowing budding to continue until mutually complementary surviving parts have been segregated from one another. Detailed studies of cell lineage in irradiated yeast might be very instructive.

*Modifying Effects on Ultraviolet Action.* Various factors have been studied which modify the effects of ultraviolet radiation on fungi; some of these are listed in Table 11-5. Perhaps the most important is the marked

ameliorating effect of visible light applied following irradiation with ultraviolet, a phenomenon which is fully discussed by Dulbecco (Chap. 12 of this volume). This has been shown for several kinds of ultraviolet effects on numerous organisms including the killing of *Streptomyces griseus*, *P. notatum*, and *S. cerevisiae* (Kelner, 1949a, b), killing and mutation of *N. crassa* (Goodgal, 1949), reverse mutation of inositolless *Neurospora* (Brown, 1951), killing and mutation of haploid and diploid *Saccharomyces* (Pomper, unpublished), and suppression of enzymatic adaptation in *Saccharomyces* (Swenson, 1950). Photoreactivation provides a striking demonstration of the existence of intermediate states between the absorp-

TABLE 11-5. FACTORS WHICH MAY ALTER THE EFFECTIVENESS OF ULTRAVIOLET

Factor	Organism	Remarks	Reference
Visible light.....	Various fungi	Reverses killing and mutation	Kelner, 1949a, b
Pretreatment with nitrogen mustard	<i>Aspergillus terreus</i>	Increases mutation rate	Swanson and Goodgal, 1948
Pretreatment with nitrogen mustard	<i>Neurospora crassa</i>	Increases mutation rate	Swanson <i>et al.</i> , 1949
Pretreatment with dinitrophenol	<i>Aspergillus terreus</i>	Increases mutation rate	Swanson and Goodgal, 1950
Methylcholanthrene	<i>Saccharomyces cerevisiae</i>	Sensitization to longer ultraviolet	Hollaender <i>et al.</i> , 1939
Pretreatment with infrared radiation	<i>Aspergillus terreus</i>	Slight increase in mutation rate	Swanson <i>et al.</i> , 1948
Postheating.....	Yeast	Increased killing	Anderson and Duggar, 1941
Age of cells.....	Various fungi	Varying results	Dimond and Duggar, 1941; Oster, 1934b; Dickson, 1932
Temperature at time of exposure	Yeast	Irradiation less efficient in killing in cold than at higher temperatures	Oster, 1934b

tion of ultraviolet quanta and their final biological effects, and it is economical to suppose that killing, mutation, and impaired enzymatic adaptation are mediated through similar mechanisms at the stage where photoreactivation occurs. It should be emphasized that all work reported with ultraviolet radiation must be scrutinized carefully to see whether or not the results have been influenced in an unrecognized manner by the effects of visible light. This is especially true of material published before 1949, when the photoreactivation phenomenon was so clearly demonstrated (Kelner, 1949a).

Other factors or agents have been considered which might influence the sensitivity of microorganisms to ultraviolet radiation. Nitrogen mustard,



in submutagenic concentration when administered with ultraviolet radiation (2537 Å), has been found to give an increase in mutation rate of *A. terreus* of 300–400 per cent over that caused by the irradiation alone (Swanson and Goodgal, 1948). This work was limited to observation of morphological variants. A microconidial strain of *Neurospora* has been employed, both morphological and nutritionally exacting mutants being scored, in similar experiments leading to the same results, i.e., an increase in mutagenicity of ultraviolet radiation after a 30-min pretreatment with 0.1 per cent aqueous solution of nitrogen mustard (Swanson *et al.*, 1949). Pretreatment with dinitrophenol also increased the morphological mutation rate of *A. terreus*, whereas potassium cyanide had no effect (Swanson and Goodgal, 1950). Posttreatment with dinitrophenol and submutagenic levels of nitrogen mustard also increased the mutagenic effectiveness of the radiation, suggesting to these authors an indirect action of the radiation. An interpretation based on direct action is, of course, equally tenable, since in our present state of ignorance it is as reasonable to assume semistable intermediate states of the genic material as extragenic chemical intermediates.

A photodynamic sensitization by methylcholanthrene of *S. cerevisiae* to light in the 3450–4500 Å range has been noted (Hollaender *et al.*, 1939). Pretreatment with infrared radiation has been found to raise slightly the mutation frequency at high doses of ultraviolet with *A. terreus* (Swanson *et al.*, 1948). Prior exposure to heat did not sensitize yeast cells to the lethal action of subsequent ultraviolet radiation, although reversal of the sequence did have a sensitizing effect (Anderson and Duggar, 1941). Older yeast cells have been found to be more resistant to killing than younger cells (Oster, 1934b) and a similar result has been reported for the spores of *Rhizopus suinus* (Dimond and Duggar, 1941). There appears to be a slight temperature effect with yeast, since for the same killing more energy is required at 8°C than at higher temperatures (Oster, 1934b). No effect of humidity was noted with *A. niger* spores (Zahl *et al.*, 1939). The significance of such isolated observations remains obscure, but it is likely that satisfactory interpretations and comparisons of experimental data have been greatly hindered by lack of awareness of the influence of seemingly irrelevant factors on the final outcome. There is a very considerable need to explore more thoroughly and under controlled genetic conditions the effects of age, nutritional state, and other environmental factors upon the sensitivity of cells exposed to ultraviolet radiation.

### IONIZING RADIATIONS

The ionizing radiations are free from the dependence on specific absorption which is an outstanding characteristic of ultraviolet. The elementary physical processes which they bring about within the cells involve

much greater energies than in the case of ultraviolet. The much milder processes brought about by ultraviolet or by photodynamic action of even longer wave lengths are entirely adequate, however, to produce the same biological effects which are apparently caused by ionizing radiation. It is to be expected, then, that comparison of the biological effects of different radiations solely on the basis of their energies is singularly uninformative. The major differences are found in comparisons of kinetic experiments with the different radiations, and of environmental modifying factors. Thus the survival of *Neurospora* microconidia is exponential with ultraviolet (Norman, 1951), but is complex with a higher order component with X rays (Giles, 1951). In yeast the order of the survival curves is similar to the ploidy with X rays, but much higher with ultraviolet (Pomper, unpublished; Sarachek and Lucke, 1953). Photoreactivation, the strongest modifying factor with ultraviolet, is not observed with X rays, whereas oxygen tension during irradiation, the strongest modifying factor with X rays, is not influential with ultraviolet.

Various general effects of ionizing radiations on fungi have been reported. With yeast, no stimulation of growth was observed at low doses with X or  $\alpha$  rays (Lacassagne and Holweck, 1930). Cell division of yeasts was retarded (Holweck and Lacassagne, 1930a). After higher dosages of X rays, cells often underwent two divisions before swelling up and dying (Holweck, 1930; Wyckoff and Luyet, 1931; Holweck and Lacassagne, 1930a; Brace, 1950; Henshaw and Turkowitz, 1940). The phenomenon of delayed death has been emphasized (Holweck, 1930; Wyckoff and Luyet, 1931) as a major difference between ultraviolet and X radiation but Oster (1934b) has observed the delay following ultraviolet. It has been reported that some enlarged cells obtained after X irradiation or exposure to radium have continued in culture as enlarged cells (Brace, 1950; Bauch, 1943). A suggestion of induced polyploidy has been made but cannot be accepted without genetic proof. Experiments with  $\alpha$  rays (Lacassagne, 1930) and cathode rays (Wyckoff and Luyet, 1931) suggest that death occurs after a cell division. X-irradiated spores of *Chaetomium cochliodes* all germinated but only a fraction survived to maturity (Dickson, 1932).

Changes in the sexual pattern of certain yeasts and molds have also been reported. In a culture of normally isogamic *Mucor genevensis*, frequent heterogamic conjugations were observed after X irradiation (Nadson and Philippov, 1925). Two strains of this mold were isolated, one showing an increased amount of zygote formation with a decrease in sporangia, the second the reverse. *Zygorhynchus molleri* also exhibited the latter behavior (Nadson and Philippov, 1928b). The yeast *Nadsonia fulvescens*, normally heterogamic, responded to X rays by developing a series of abnormal sexual reactions, leading finally to complete loss of sexuality with greater doses (Nadson and Philippov, 1926).



*Comparison of Different Ionizing Radiations.* The dissipation of energy along the paths traversed by ionizing particles, rather than randomly throughout the material, is undoubtedly the major reason for differences in biological effectiveness of physically equivalent doses of ionizing radiations of different quality (Lea, 1947). Some studies on the comparative effectiveness of different ionizing radiations are summarized in Table 11-6. With *A. terreus* as test organism, the interesting observation has been made that densely ionizing radiations (neutrons and  $\alpha$  rays) were more effective in killing but less effective in mutating than  $\gamma$  and X rays (Stapleton and Martin, 1949). A further difference has been observed in that survival followed a sigmoidal curve whereas mutation production was linear with dose (Stapleton and Martin, 1949). *Aspergillus niger* spores behave similarly to *A. terreus*, as regards comparative lethal effectiveness of densely ionizing and more disperse radiation, while *S. cere-*

TABLE 11-6. RELATIVE EFFECTIVENESS OF IONIZING RADIATIONS IN CAUSING KILLING AND MUTATION IN FUNGI

Organism	Effect	Order of decreasing effectiveness	Reference
<i>Aspergillus terreus</i> . .	Killing	Neutrons and $\alpha$ rays > X rays and $\gamma$ rays	Stapleton and Martin, 1949
<i>Aspergillus terreus</i> . .	Mutations	$\gamma$ rays > X rays > neutrons and $\alpha$ particles	Stapleton and Martin, 1949
<i>Aspergillus niger</i> . . . .	Killing	Dense ionization track > disperse	Zirkle, 1940
Yeast . . . . .	Killing	$\alpha$ rays > X rays	Zirkle, 1940; Tobias, 1952
Yeast . . . . .	Killing	X rays > $\alpha$ rays	Holweck, 1930

*visiae* showed only a slight effect, and *Escherichia coli* a complete reversal of effectiveness (Zirkle, 1940). There appears to be some contradiction in the data on yeast, since  $\alpha$  rays have been reported to be more effective (Tobias, 1952) and less effective (Holweck, 1930) than X rays; and longer X rays (8 A) to be less efficient in killing than shorter radiation (2 A) (Holweck and Lacassagne, 1930b). At the point of 50 per cent lethality, twice the cathode-ray dose, three times the X-ray dose, and six times the ultraviolet exposure are required for *Rhizopus nigricans* spores, as compared to yeast cells (Luyet, 1932). In general, such differences between the effectiveness of radiations of different quality are indicative of a rather close localization of the effects to points along the paths of ionizing particles. If the effects were not localized, it would be impossible to explain the results in terms of the spatial relations of the biological targets and the nonrandom distribution of elementary processes. Present knowledge provides no reasonable alternative.

*Dose-effect Curves.* Survival curves have been obtained with various fungi, a few of which are listed in Table 11-7. The survival curves

TABLE 11-7. TYPES OF SURVIVAL CURVES OBTAINED WITH X RAYS FOR A FEW FUNGI

Organism	Type of curve	Remarks	Reference
<i>Saccharomyces cerevisiae</i> (diploid)	Sigmoidal	Exponential curve has been reported (Henshaw and Turkowitz, 1940)	Wyckoff and Luyet, 1931; Latarjet and Ephrussi, 1949
<i>Saccharomyces cerevisiae</i> (haploid)	Exponential		Latarjet and Ephrussi, 1949
<i>Torulopsis cremoris</i> . . . .	Exponential	No genetic analysis	Anderson and Turkowitz, 1941
<i>Chaetomium globosum</i> . . .	Sigmoidal	Five hits to kill	Ford and Kirwan, 1949
<i>Rhizopus nigricans</i> . . . . .	Sigmoidal		Luyet, 1932
<i>Streptomyces flavocolus</i> . . .	Exponential		Kelner, 1948
<i>Streptomyces griseus</i> . . . .	Exponential		Savage, 1949
<i>Aspergillus terreus</i> . . . . .	Sigmoidal		Stapleton and Martin, 1949

reported for yeast exposed to X rays have been sigmoidal with diploid *S. cerevisiae* (Wyckoff and Luyet, 1931; Latarjet and Ephrussi, 1949) but exponential with a haploid strain (Fig. 11-6). Independent confirmatory results have also been obtained, showing that, with ionizing radiations, a haploid yeast is killed logarithmically, a diploid sigmoidally, and a tetraploid also sigmoidally (Pomper, unpublished; Tobias, 1952; Lucke and Sarachek, 1953). An exponential survival curve has been reported for presumably diploid *S. cerevisiae* exposed to X rays (Henshaw and Turkowitz, 1940), and here a genetic analysis of the presumed diploid would be a helpful datum in appraisal. Tobias (1952) has reported that certain stocks obtained from originally diploid cells surviving X irradiation were found to have survival curves differing from those of their diploid progenitors in being more nearly exponential. One is tempted to interpret this finding as indicative of the loss of a large portion of the genetic appa-

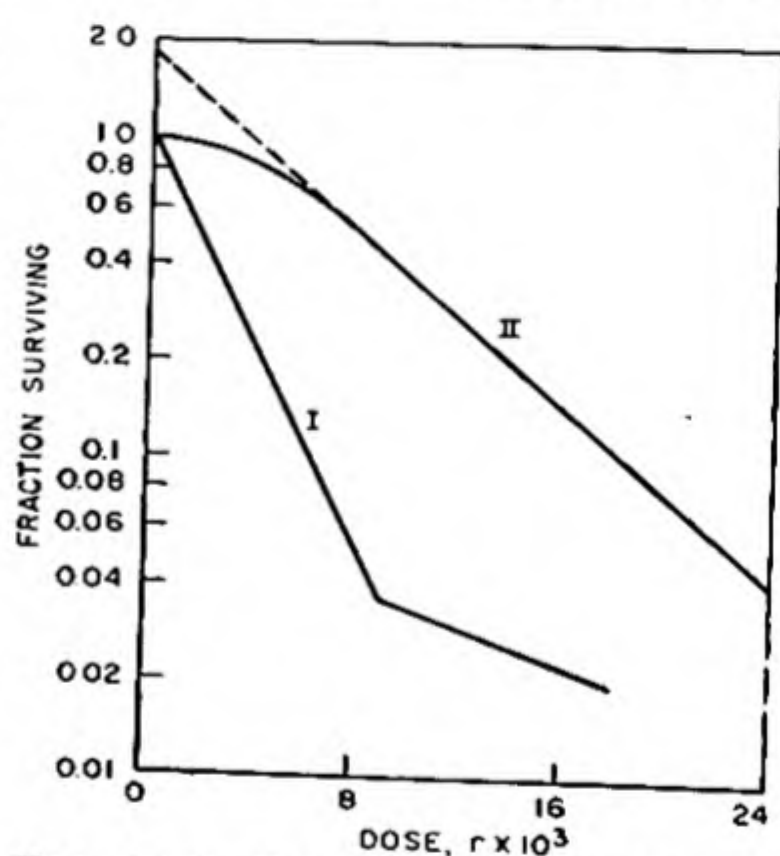


FIG. 11-6. Survival of haploid and diploid *S. cerevisiae* exposed to X rays. The inactivation of yeast cells by X rays. Curve I, haploid cells; curve II, diploid cells. (The data, from Latarjet and Ephrussi, 1949, were adapted by Norman.)



ratus, leaving the initially diploid cell in a condition more nearly approximating the haploid. Experiments with genetically marked diploids would be especially informative on this point.

There has been considerable confusion in interpreting sigmoidal survival curves, particularly with regard to determining the number of events necessary to kill. The difficulty arises largely from failure to recognize that one must distinguish between two completely different meanings of the number of events: (1) number of hits that are necessary, and (2) number of units that must be inactivated. The difference between these interpretations and the reasons for preferring the latter in most cases have been discussed by Atwood and Norman (1949).

A study of the survival kinetics in material in which the mitotic stage, the ploidy, or the number of nuclei per cell can be systematically varied will be most profitable. The lack of such criteria precludes, in our present rudimentary state of knowledge, any satisfactory interpretation of survival data, and for this reason most of the experimental observations in Tables 11-4 and 7 must be simply noted without comment.

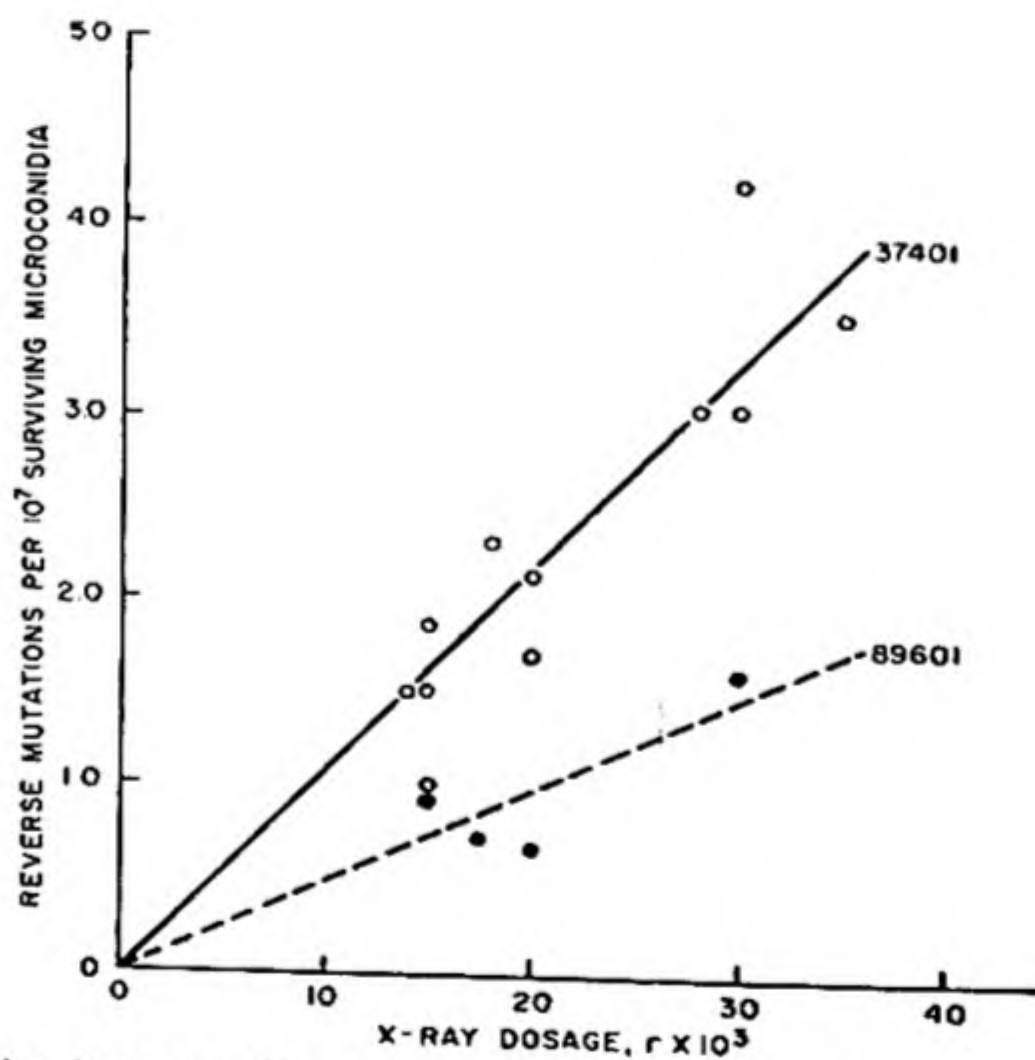
Curves relating mutation production to radiation dosage have been obtained for X rays with *A. terreus* (Stapleton and Martin, 1949), *N. crassa* (Sansome *et al.*, 1945), and *P. notatum* (Hollaender and Zimmer, 1945), with morphological mutations as the criterion, mutation being essentially proportional to dose in all cases. *Streptomyces flaveolus* differed in that the curve broke consistently downward between 100,000 and 200,000 r (Kelner, 1948). A critical study of mutation frequency is that recently carried out with *Neurospora* (Giles, 1951) using reversions to inosital independence as assay procedure. A linear relation between dose and frequency of mutations was obtained with X rays, as shown in Fig. 11-7.

*Modifying Factors.* Certain environmental or cultural conditions have been found to exercise a considerable effect in modifying results obtained with ionizing radiations (Table 11-8). Rather striking effects have been obtained, both in augmenting and in decreasing the magnitude of the results expected from a given dosage. Pretreatment with infrared radiation has been found to increase significantly the morphological mutation rate with *A. terreus* and *Trichophyton mentagrophytes* (Swanson *et al.*, 1948; Hollaender and Swanson, 1947). The range reported as active was 7000–18,000 Å, with a maximum at 10,000 Å.

Heat had no saltating effect on *Chaetomium cochliodes* (Dickson, 1932). That temperature may play an important role is suggested by the observation with *Saccharomyces ellipsoideus* of a reduction in killing from 37 to 19 per cent at 13,000 r by holding the cells on ice for 10 days after irradiation (Latarjet, 1943). This observation led to the suggestion that the radiation damage may actually be caused in large part by the secondary chemical reactions occurring after irradiation, rather than the primary,

TABLE 11-8. FACTORS WHICH MAY ALTER X-RAY EFFECTS

Factor	Organism	Effect	Reference
Pretreatment with infrared radiation	<i>Aspergillus terreus</i> and <i>Trichophyton mentagrophytes</i>	Increase mutation rate	Swanson <i>et al.</i> , 1948; Hollaender and Swanson, 1947
Postincubation at reduced temperatures	<i>Saccharomyces ellipsoideus</i>	Decrease killing (in some experiments)	Latarjet, 1943
Age of culture.....	<i>Saccharomyces ellipsoideus</i>	Older cells more easily killed	Lacassagne and Holweck, 1930
Age of culture.....	<i>Chaetomium cochliodes</i>	Older mycelium higher mutation rate	Dickson, 1932
Moisture content...	<i>Saccharomyces cerevisiae</i>	Dried cells higher survival	Dunn <i>et al.</i> , 1948
Moisture content...	<i>Aspergillus terreus</i>	Dried spores higher survival	Stapleton and Hollaender, 1952
Absence of oxygen..	<i>Aspergillus terreus</i>	Reduction of killing	Stapleton and Hollaender, 1952
Absence of oxygen..	<i>Torulopsis cremoris</i>	Reduction of killing	Anderson and Turkowitz, 1941

FIG. 11-7. Relation between X-ray dosage and frequency of reverse mutations in two inositolless mutants (in *c m f* stocks). (Giles, 1951.)



temperature-insensitive reaction, and that the effect of the cold treatment was to slow down these injurious reactions so that the cell might recover (Latarjet, 1943). Another paper notes no effect on this same yeast of holding cells on ice for several days after X or  $\alpha$  irradiation (Lacassagne and Holweck, 1930).

Old resting cells of *S. ellipsoideus* have been found to be more sensitive than young dividing cells (Lacassagne and Holweck, 1930; cf. Oster, 1934b). Time of irradiation apparently has little effect (Lacassagne and Holweck, 1930), although one might expect that too prolonged an exposure might lead to experimental complications. Toxic substances formed in the suspending medium might play an important role if experiments are prolonged, although under ordinary circumstances this has not been found to be a serious factor.

Experiments in which two other variables, oxygen and humidity, have been shown to be important in modifying the lethal effects of X rays have aroused considerable interest. Dried *S. cerevisiae* was found to be more resistant to X rays than normal (wet) cells (Dunn *et al.*, 1948). Wet spores of *A. terreus* were more susceptible to the killing action of X rays than dry spores (Stapleton and Hollaender, 1952). X rays kill spores of *A. terreus* more efficiently in the presence of oxygen than in its absence (Stapleton and Hollaender, 1952). A similar observation had earlier been made with the yeast *Torulopsis cremoris*; viz., in the absence of air a greater dosage was necessary to bring about the same killing action (Anderson and Turkowitz, 1941). It was observed that this yeast, if grown without shaking or aeration and then irradiated, was more resistant than if grown similarly but shaken prior to irradiation. The observation takes on added validity in view of recent results showing that the oxygen effect is general.

*Conclusions.* In summary, it may be said that fungi, in common with other organisms, may be killed or mutated by various radiations and that these effects may be intensified or decreased by adjunctive treatments. The results of some of the adjunctive treatments are most easily interpreted as influencing the indirect radiation action, e.g., the chemical intermediates produced by radiation which subsequently react with the biologically significant materials. On the other hand, the differences in effectiveness of ionizing radiations of different quality are most easily interpreted in terms of sharply localized effects. These conditions are not, of course, mutually exclusive.

It seems reasonable to ascribe a large role to events in the nucleus, based on the observations of haploid and diploid yeast survival curves (Latarjet and Ephrussi, 1949; Tobias, 1952; DeLong and Lindegren, 1951; Pomper, unpublished; Lucke and Sarachek, 1953), and on the differences between uni- and multinucleate conidia of *Neurospora* (Norman, 1951). The relation, if any, between these nuclear effects and mutation per se

is not known. From a phenogenetic point of view it is difficult to decide what is meant by dominant lethal mutations in microorganisms. Recessive lethal mutations are not, by definition, lethal except in haploid, effectively uninucleate organisms. Furthermore, it is known from studies with various organisms that radiation (especially at higher doses) may cause cytoplasmic changes, and so the role of the cytoplasm, although probably of secondary importance, cannot be disregarded. Experimental means of approaching such problems are rapidly developing as a result of intensified research in microbial genetics, and the application of these new methods may be expected to dispel much of the confusion which now predominates in this area of radiation biology.

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## CHAPTER 12

### Photoreactivation

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*Introduction. Historical note. Photoreactivation in bacteriophages: Conditions of inactivation—Methods for studying photoreactivation—Adsorption of phage on bacteria a necessary prerequisite for photoreactivation—Comparison of survival curves after ultraviolet treatment in presence and in absence of photoreactivation—Kinetics of photoreactivation in phage T2 in condition of single infection—Kinetics of photoreactivation of phage T3—Kinetics of photoreactivation of phage T2 in condition of multiple infection—Action spectrum of the photoreactivating light—Action of chemical substances on photoreactivation—Photoreactivation of bacteriophages inactivated by agents other than ultraviolet—Photoreactivation of the induction process of phage carried lysogenically. Photoreactivation of plant viruses. Photoreactivation of bacteria: Conditions under which photoreactivation occurs—Effect of the growth stage of bacteria on photoreactivation—Comparison of survival curves after ultraviolet treatment in presence and absence of photoreactivation—Kinetics—Action spectrum of the photoreactivating light—Chemical actions connected with photoreactivation—Action of photoreactivation on the induction of mutation by ultraviolet in bacteria. Photoreactivation in Streptomyces and fungi. Photoreactivation in yeast. Photoreactivation in protozoa. Photoreactivation in echinoderm zygotes and gametes. Photoreactivation in salamander larvae. Photoreactivation in higher plants. Conclusions and summary. References.*

#### 1. INTRODUCTION

The term "photoreactivation" designates the phenomenon in which changes produced in different types of organisms, mainly microorganisms, by ultraviolet radiation of wave lengths around 2500 Å can be counteracted if the irradiated organisms, under proper conditions, are exposed to a radiation of longer wave length, in the range between 3300 and 4800 Å. The phenomenon received its designation from the fact that it was first noted as a reversal of the *inactivation* produced by the ultraviolet radiation in some microorganisms (fungi, yeasts, bacteria, bacteriophages); however, it includes reversal of effects of ultraviolet radiation other than inactivation, such as mutation, delay in time of onset of division in echinoderm eggs and in protozoa, decreased vigor in protozoa, and even morphological changes.

Since the outstanding feature of photoreactivation in microorganisms



is the reversal of the inactivation, the meaning of inactivation must be clarified. The definition is a relative one: a microorganism after treatment with a radiation is inactive if in given standard conditions it is not able to give rise to indefinite multiplication. This is tested by determining whether or not indefinite growth can be obtained by placing the organism immediately after irradiation in darkness and at optimum temperature into a nutrient medium. Growth is considered absent if no visible colony is formed. A fraction of the organisms judged inactive by this technique may be judged active if the technique is changed; examples are known in bacteria in which the type of growth medium used or the temperature of incubation affects the fraction of organisms able to give rise to detectable growth. In bacteriophages the criterion is still more imperfect since an active phage particle can develop a colony only if the first sensitive bacterium infected by it bursts within a given time of incubation. In spite of this imperfection, a fairly precise method for the study of reactivation phenomena can be developed by strict standardization of technique.

## 2. HISTORICAL NOTE

Photoreactivation was discovered and its generality appreciated by Kelner (1949a), who observed reversal by visible light of ultraviolet inactivation in spores of *Streptomyces griseus*. Previous observations of the phenomenon can be found in the older literature, the most pertinent of which is an observation by Whitaker (1941-42) on the ability of visible light to counteract the action of ultraviolet on *Fucus* eggs. A review of older observations has been given by Kelner (1950b).

At this time the chemical and biological mechanisms involved in ultraviolet effects and in photoreactivation are very imperfectly understood, and the observations that have been reported for various organisms cannot yet be discussed from a unified point of view. Ultraviolet can affect any substances which absorb the radiation. To this class of substances belong the nucleic acids, the proteins, and numerous smaller molecules with specific functions. There have been no reports of photoreactivation of enzymes inactivated by ultraviolet; however, the following significant observation has been published by Shugar (1951). Crystalline triose-phosphate dehydrogenase, which is firmly combined with diphosphopyridine nucleotide (DPN), is partially inactivated during preparation, owing to oxidation of essential SH groups, and can be reactivated by exposure to light of the near-ultraviolet spectrum, with a peak at 3400 Å, which is therefore in the absorption band of reduced DPN. The mechanism of reactivation could be a reduction of S—S groups consequent to light absorption in contiguous reduced DPN molecules. Here are found several features which occur in photoreactivation: the spectral range involved is similar; only a fraction of the enzyme activity can be reac-

tivated, only the S—S groups contiguous to reduced DPN molecules being reduced; and reactivation is a reaction of approximately first order.

Reactivation of triosephosphate dehydrogenase by light may well be a model for photoreactivation. The latter phenomenon differs apparently because it affects only functional ultraviolet changes related to growth, the simplest of which is probably the formation of adaptive enzymes (Swenson and Giese, 1950); however, this may be so because photoreactivation is an indirect process, involving substances commonly found in the cytoplasm of cells, and because the most outstanding damages produced by ultraviolet are those affecting growth.

In this review the observations made on different organisms will be discussed separately. By far the most detailed quantitative measurements have been made with the coli phages of the T series. These will be presented first, and some theoretical notions verifying these data will be developed. Measurements of photoreactivation in bacteria are almost as detailed as those on phage, and these data, too, can be accounted for on the basis of a few simple assumptions. However, it is remarkable that the formal schemes developed for photoreactivation in phage and bacteria are quite different and seemingly incompatible. Since it does not seem reasonable to assume radically different mechanisms for so obviously similar phenomena, both theories must be viewed with suspicion, and a deeper interpretation of all the data must be sought. In the remainder of this review the scattered data which have been reported for other organisms will be briefly summarized.

### 3. PHOTOREACTIVATION IN BACTERIOPHAGES

Photoreactivation has been studied in the bacteriophages of the T group active on *Escherichia coli* strain B (Dulbecco, 1949, 1950).

#### 3-1. CONDITIONS OF INACTIVATION

The bacteriophages used are very stable, and can be kept for months as suspensions in buffered saline solutions. Such suspensions are inactivated by exposure to an ultraviolet source, which in the majority of the experiments has been a G.E. germicidal lamp, emitting 80 per cent of its energy in the wave length 2537 Å.

After irradiation a certain fraction of the particles is inactive. The logarithm of the fraction that is still active after a given ultraviolet dose, plotted versus the dose, gives the so-called "survival curve," whose shape is characteristic for a given phage.

#### 3-2. METHODS FOR STUDYING PHOTOREACTIVATION

If equal samples from a suspension of phage irradiated with ultraviolet are plated with sensitive bacteria on two equal plates and one is incubated



in darkness and the other under a source of white light, the plate kept in the light shows after incubation a much greater number of bacteriophage colonies; this is the basic experiment of photoreactivation of bacteriophages. A similar experiment can be done by mixing bacteria and bacteriophages in a nonnutrient medium, usually a buffer solution, so that adsorption of the bacteriophages onto the sensitive bacteria can take place, but not growth. The mixture is exposed to a white light and then plated with sensitive bacteria on a plate of nutrient agar, which is incubated in darkness.

Both these methods are useful. The first has been called the method of photoreactivation *on the plate*; the second, the method *in liquid*. The choice of the method to be used depends on the type of information sought. The method on the plate is used for orientation experiments, for testing for qualitative effects, and for some quantitative studies, particularly with phages which adsorb slowly (e.g., T1 and T5). The method in liquid is generally used in quantitative work; it has been successively applied to bacteriophages T2, T3, T4, and T6.

### 3-3. ADSORPTION OF PHAGE ON BACTERIA A NECESSARY PREREQUISITE FOR PHOTOREACTIVATION

Free phages, inactivated with ultraviolet and then exposed to the photoreactivating light *in the absence of bacteria*, are not reactivated; for photoreactivation to occur the particles must be adsorbed on the sensitive bacteria. Demonstration that adsorption is required for photoreactivation has been obtained by mixing sensitive bacteria and irradiated bacteriophages under conditions which do not permit adsorption, exposing the mixture to light, and then plating a sample from the mixture; the fraction of active particles remains unchanged. The condition for non-adsorption can be realized either by suspending phage and sensitive bacteria in a solution which has an ionic composition unfavorable for adsorption or by mixing a phage with cells of a mutant of *E. coli* B resistant to the phage (e.g., phage T1 and bacteria B/1).

Two types of adsorption of bacteriophage particles on the sensitive bacteria occur, depending on the ionic strength of the medium: when the ionic concentration of the medium is too low, adsorption is reversible—the adsorbed phage can be eluted with distilled water (Puck *et al.*, 1951); at high ionic concentration adsorption becomes irreversible. Photoreactivation takes place only if the phage has been adsorbed irreversibly (Dulbecco, unpublished).

Bacteriophages adsorbed on bacteria in a medium which does not permit growth remain perfectly photoreactivable over a period of several hours. The medium may be either a buffer, in which case the bacteria are taken from a logarithmic phase culture, washed and resuspended in the buffer, or a synthetic medium containing a limited amount of glucose

as the sole carbon source, in which the bacteria have grown to complete exhaustion of the sugar. If, on the contrary, the bacteriophages are adsorbed on bacteria in a medium supporting growth, the photoreactivability of the phages decreases with time elapsed between infection and exposure to the photoreactivating light. For example, for bacteriophage T2 adsorbed on *E. coli* B at 37°C in beef extract, very little or no photoreactivation can be obtained if exposure to white light is started 30 min after infection.

Phage inactivation is obtained also by irradiating bacteria infected with active bacteriophages (Luria and Latarjet, 1947); also in this case photoreactivation can be obtained (S. Benzer, personal communication). Photoreactivation is also obtained if the inactive phage is adsorbed on sensitive bacteria previously irradiated with ultraviolet.

#### 3-4. COMPARISON OF SURVIVAL CURVES AFTER ULTRAVIOLET TREATMENT IN PRESENCE AND IN ABSENCE OF PHOTOREACTIVATION

Survival curves (see Sect. 3-1) of different phages in absence of photoreactivation are of two types: for the large phages (T2, T4, T5, T6) they have an initial curvature with downward concavity and then tend to straight lines; for the small phages (T1, T3, T7) they are straight near the origin and then decrease in slope, tending again to straight lines (Dulbecco, 1950).

Survival curves obtained after the irradiated samples have been exposed to photoreactivation have similar shapes as the curves in darkness, but they are shifted in the direction of a higher survival.

The comparison of the curves obtained for a given phage in darkness with those obtained after maximum photoreactivation shows that if the ultimate straight parts of the two curves are extrapolated back toward the origins, they intersect in the proximity of the abscissa. For the larger phages, which have a curvature with downward concavity, the intersection point lies to the right of the origin. For the small phages it lies to its left (Fig. 12-1) (Dulbecco, unpublished).

The reason for this behavior is not yet clear. In the section on bacterial photoreactivation (Sect. 5-3) it will be shown that in bacteria a different relation between survival curves in dark and after photoreactivation has been observed, a relation which has been called the "principle of constant ultraviolet dose reduction." It should be emphasized here that the same relation does not hold in bacteriophage reactivation. The methods for determining the existence of a constant dose reduction will be discussed in Sect. 5-3, where the explanation of Fig. 12-1, in which the two tests for such a determination have been applied to phage, both with negative results, is also given.

For every phage the survival curves in darkness and after maximum



photoreactivation tend to straight lines with different slopes. The slope is an important quantitative datum. In fact, if a survival curve of an irradiated population is a straight line, its slope determines the cross section for the radiation of the individual elements. In multiple-hit curves the slope of the straight line to which the curve tends at high doses of the radiation has the same meaning; in an unhomogeneous population the final slope measures the cross section of the individuals of the class

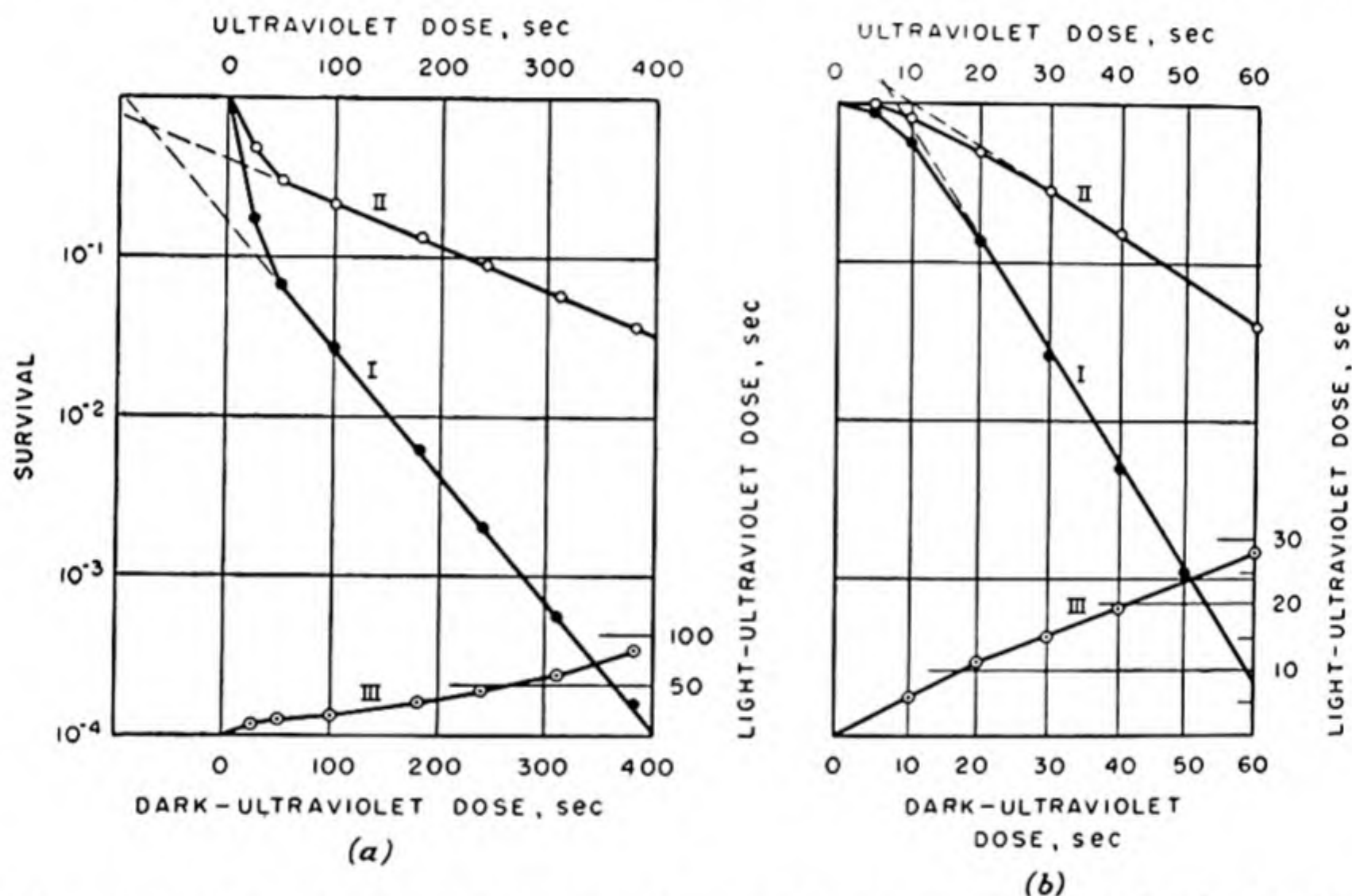


FIG. 12-1. Survival curves in darkness and after maximum photoreactivation for (a) phage T1 and (b) phage T2. Curves I are for darkness; II, after maximum photoreactivation; III, curve giving the ultraviolet dose corresponding to a given survival after photoreactivation versus the dose corresponding to the same survival in darkness (see Sect. 5-3). For survival curves, log survival (left-hand scale) is plotted versus the ultraviolet dose in seconds of exposure (upper scale). For lower curves, light-ultraviolet dose in seconds of exposure (right-hand scale) is plotted versus dark-ultraviolet dose (bottom scale).

with least cross section. The fact that in phages the survival curves in darkness and after maximum photoreactivation have different final slopes can be taken as a demonstration that the cross section of the particles to ultraviolet is reduced after photoreactivation. This means that the damage produced by ultraviolet in bacteriophages can be divided into two classes, one completely photoreactivable and the other nonphotoreactivable, with a constant ratio, independent of the ultraviolet dose. Existence of these two classes of ultraviolet damages can be explained under two different assumptions: either ultraviolet light produces two different types of chemical effects, of which only one is photoreactivable; or only

one type of damage is produced but not all the bacteriophage particles are exposed to the photoreactivating mechanism.

The cross section of phage particles to ultraviolet can thus be subdivided formally into two *sectors*, only one of which is photoreactivable. The nonphotoreactivable sector is measured by the ratio

$$1 - a = \frac{\text{slope of straight part of survival curve after photoreactivation}}{\text{slope of straight part of survival curve in darkness}}.$$

The photoreactivable sector  $a$  may be taken as a measure of the photoreactivability of different phages. Data for the seven phages of the T group adsorbed on *E. coli* B are given in Table 12-1. It is interesting to

TABLE 12-1. PHOTOREACTIVITY OF THE PHAGES OF THE T GROUP  
Photoreactivable Sector  $a$

Phage	of Cross Section
T1	0.68
T2	0.56
T3	0.39
T4	0.20
T5	0.20
T6	0.44
T7	0.35

note that among the related phages T2, T4, and T6, phage T4, which is much less photoreactivable, apparently contains less nucleic acid than the other two (Luria *et al.*, 1951). A similar correlation occurs also in plant viruses (Sect. 4).

The ratio of the two sectors of the total cross section is not a characteristic of the phage alone, but is somewhat affected by the host bacterium in which photoreactivation occurs. Phage T2 has a slightly higher photoreactivable sector if adsorbed on *E. coli* B/1 than if adsorbed on *E. coli* B (Dulbecco, unpublished).

### 3-5. KINETICS OF PHOTOREACTIVATION IN PHAGE T2 IN CONDITION OF SINGLE INFECTION

3-5a. *Effect of Different Doses of Photoreactivating Light at Constant Intensity.* A sample of ultraviolet-irradiated phage is adsorbed on sensitive bacteria in darkness and then exposed to a photoreactivating light of constant intensity; at regular time intervals samples are taken and assayed for active phage. The number of active particles,  $p(t)$ , increases with the time of illumination; if exposure is continued for a long time a maximum number of active particles,  $p(\infty)$  is reached, after which any further illumination is without effect. In experiments made with the same ultraviolet-irradiated samples with reactivating light of different intensities, approximately the same maximum is reached, as shown in



Fig. 12-2, but in different times, shorter times being required at higher intensities, within certain limits (see also Sect. 3-5b).

If the light intensity is very high, continuation of the illumination after the maximum is reached results in loss of infective centers; this effect is not specific for the photoreactivated particles, since it can be obtained also when bacteria infected with *active* particles are exposed to a photoreactivating light of high intensity. Therefore a damaging effect of the photoreactivating light on the phage-bacterium complex interferes with the process of photoreactivation. This damage of the phage is not due to

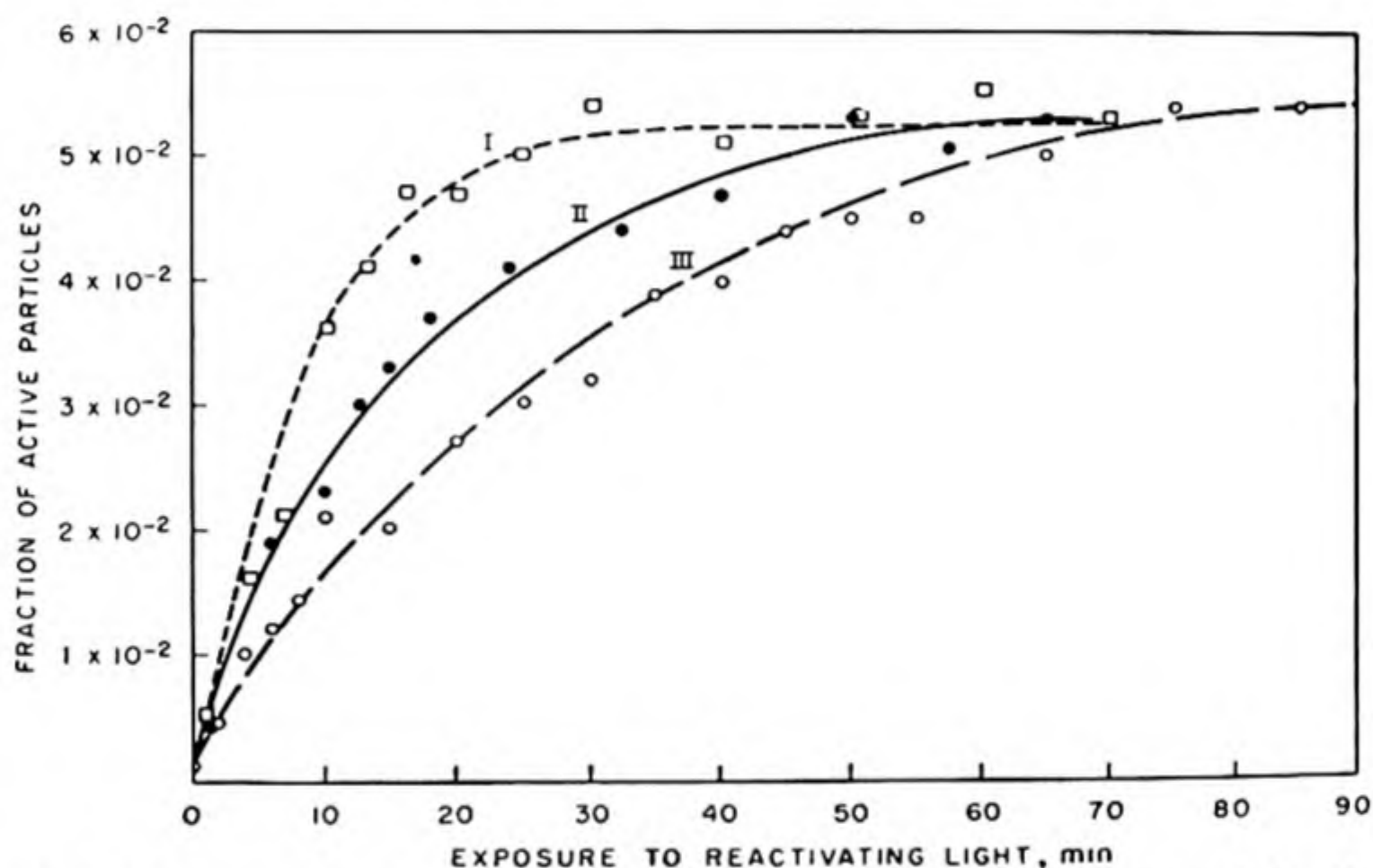


FIG. 12-2. Fraction of active particles in phage sample as a function of the time of illumination and of the light intensity. Phage T2 was irradiated for 20 sec with the germicidal lamp, adsorbed on resting bacteria, and illuminated in liquid at 37°C. Curve I was obtained with a light of intensity 10 (in arbitrary units), curve II with a light of intensity 2.9, and curve III with a light of intensity 0.6. (Dulbecco, 1950.)

the light inactivation of the bacteria (Hollaender, 1943). This requires still higher doses (Dulbecco, unpublished). The damaging effect can be reduced by reducing the intensity of the reactivating light; this, as shown later, affects photoreactivation only to a small extent, but decreases the damaging effect.

The logarithm of the fraction of photoreactivable particles that have not been reactivated,  $\log [1 - p(t)/p(\infty)]$ , plotted versus the time of illumination with a light of constant intensity is called the "photoreactivation curve." In the case of phage T2 this curve is very nearly a perfectly straight line (Fig. 12-3).

Curves determined with the greatest accuracy show one and sometimes two slight deviations from linearity. The first deviation always occurs at very short periods of illumination, and is due to a relatively higher effi-

ciency of the first short period of illumination. It can be eliminated by using a flashing instead of continuous light, as will be shown in the section on the effect of interrupted light (Sect. 3-3d). A second deviation may occur at high intensities and long exposures, owing to the inactivation phenomenon mentioned previously. These two deviations find an explanation in accessory phenomena and do not affect the linearity of the curve in principle.

The linearity of the curve shows that a photoreactivable particle is reactivated by one hit of the reactivating light, or, in other words, that the transformation of photoreactivable into photoreactivated particles follows a first-order reaction. The slope of the photoreactivation curve is called

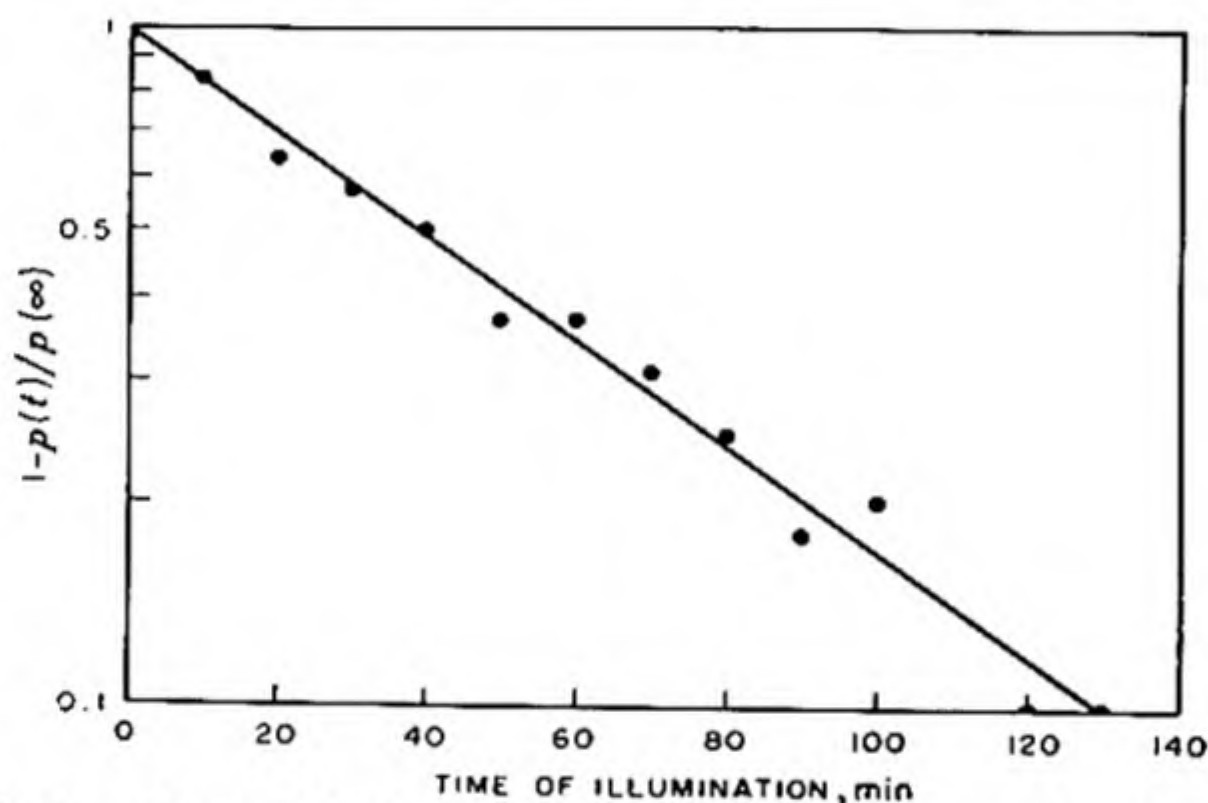


FIG. 12-3. The logarithm of the fraction of photoreactivable particles that has not been reactivated after a given time of illumination,  $1 - p(t)/p(\infty)$ , plotted against the time of illumination in minutes. Phage T2 was irradiated for 20 sec with the germicidal lamp, adsorbed on bacteria in buffer, and illuminated in liquid at 37°C. (Dulbecco, 1950.)

the "rate of photoreactivation." It measures the probability that a given photoreactivable phage particle is photoreactivated in unit time.

The fact that T2 phage particles which have received several ultraviolet hits are photoreactivated by one photoreactivating hit is an important item of information. To explain this some particular mechanism either in the inactivation or in the reactivation might be conceived. It might be considered, for example, that ultraviolet inactivation of phages consists in formation of an inhibitor on a given essential nucleotide, and that photoreactivation consists in the removal of the inhibitor. However, a T2 particle contains approximately  $5 \times 10^6$  nucleotides, and it adsorbs  $10^4$  ultraviolet quanta for each hit (M. R. Zelle, personal communication). Therefore, for each hit only one-fiftieth of the nucleotides have absorbed a light quantum. This absorption would affect the given essential nucleo-



tide only if energy can be transmitted to it from a group of 50 neighboring nucleotides or if an inhibitor localized on any nucleotide of this group can affect the essential one, for example, sterically, as well as prevent other inhibitors from localizing on the same group. Both these assumptions seem rather artificial.

Another explanation of the one-hit character of the T2 photoreactivation is to assume that photoreactivable inactivation consists of a number of independent damages, at least one per photoreactivable hit, and that one quantum of the reactivating light is able to affect them all, by some kind of trigger mechanism producing numerous molecules capable of photoreactivating. This interpretation is also very artificial.

Owing to the difficulty in interpreting T2 photoreactivation as a reversal of ultraviolet damage, a different position may be taken, and it may be considered that photoreactivation does not affect the ultraviolet damage itself, but it avoids its consequences by making available a substance normally produced in the infected bacterium, which is no longer produced as the consequence of ultraviolet inactivation. As a model, the possibility might be considered that a phage enzyme, E, acting upon a bacterial substrate A, transforms it into B, this step being essential in phage growth, and that the irradiated enzyme is not able to do so any longer. If now the system is exposed to the photoreactivating light, a photochemical product, B\*, is produced, which then produces B, and the enzymatic reaction is bypassed. In condition of continuous illumination, in which B\* is present in steady concentrations, the probability of producing B is proportional to the time of illumination, thus giving rise to the one-hit character of photoreactivation.

A final remark seems to be pertinent at this point. The one-hit character of the photoreactivation curves of this bacteriophage is derived from data extending to approximately 80 per cent of the maximum photoreactivation; in this range a one-hit curve could be produced by an inhomogeneous population composed of different classes in which the number of hits required varies from one to about ten, if the frequencies of the various classes are properly distributed; the proper distribution can be easily calculated.

3-5b. *Effect of Different Light Intensities.* Increasing the intensity of the photoreactivating light has the consequence of increasing proportionately the photoreactivation rate when the intensity is low; at higher intensities there is less increase in rate, and for very high intensities the rate tends to a maximum value (saturation). A rate-intensity plot gives a hyperbolic curve (Fig. 12-4), which follows the equation

$$R = \frac{aI}{b + cI}$$

Such a dependence shows that the rate of photoreactivation is determined

by an equilibrium condition involving a dark reaction. The substance involved in such a dark reaction might be the pigment that absorbs the light, or the product of the photochemical reaction. This point will be developed further in the treatment of "the effect of interrupted light (Sect. 3-5d).

**3-5c. Effect of Temperature.** Photoreactivation is considerably affected by temperature; this gives further evidence that dark reactions are involved. The temperature coefficient of the process ( $Q_{10}$ ) is near 2 at temperatures around  $37^{\circ}\text{C}$  and near 8 for temperatures near  $0^{\circ}\text{C}$ . This considerable variation of  $Q_{10}$  with temperature shows that more than one dark reaction is involved in the process.

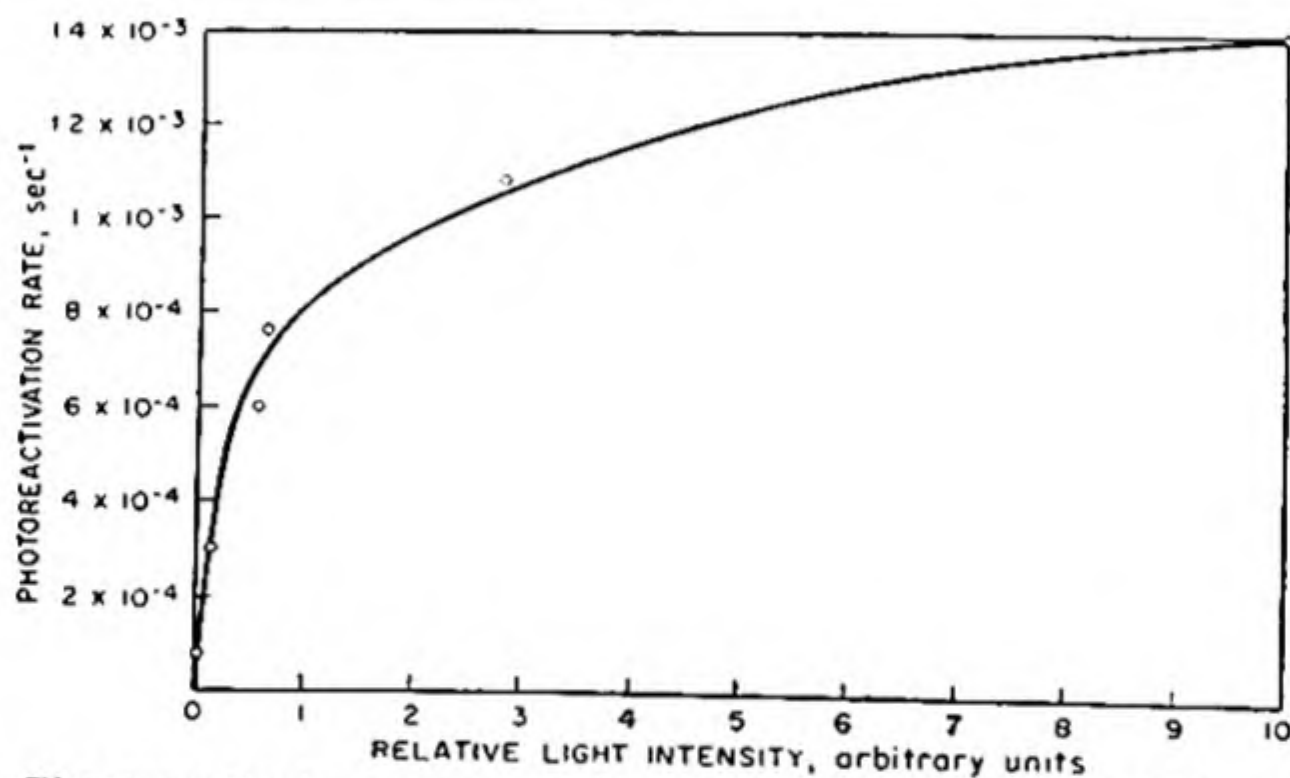


FIG. 12-4. Photoreactivation rate as a function of the intensity of the reactivating light. The photoreactivation rate is expressed in reciprocal seconds and the light intensity in arbitrary units. Phage T2 was irradiated with the germicidal lamp, adsorbed on resting bacteria, and illuminated in liquid at  $37^{\circ}\text{C}$ .

**3-5d. Effect of Interrupted Light.** The analysis of the effect of interrupted light has been carried out by Bowen (1953).

One of his most significant observations was that with a fixed dose of light given as long flashes (longer than 1 min) and long dark intervals, the fraction of reactivated particles is similar to that obtained with continuous illumination; by making the flashes shorter, without changing the total dose, a point is reached at which the reactivated fraction increases and tends to a maximum when the length of the flashes tends to zero.

Another observation was that a fixed dose of light given as short flashes of constant length has a different effect according to the length of the dark period interposed between the flashes. The number of reactivated particles increases by increasing the length of the dark periods, and for very long intervals it tends asymptotically to a limit value.

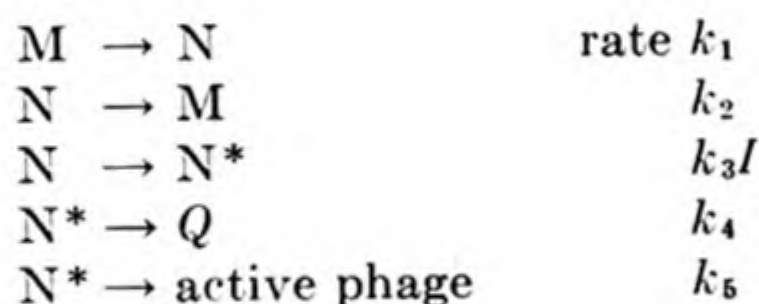
A practical consequence of these findings is that if the number of particles photoreactivated is determined as a function of the time of exposure



to a light of constant intensity, for short exposures the number is relatively higher than for longer exposures, so that the photoreactivation curve shows a deviation from linearity (Sect. 3-5a). If the same curve is obtained by giving all doses of light as series of short flashes separated by long dark periods, the deviation from linearity disappears.

The two types of experiments just described show that the amount of photoreactivation obtained with a given light dose depends not only on the light reaction, but also on dark reactions connected with it. Bowen was able to demonstrate that at least one dark reaction involved in photoreactivation must precede the light reaction, because photoreactivability of bacteria infected with inactive phage is affected by a temperature change from 40° to 0°C if the temperature of the system is changed *several minutes before* a short light flash (of 5 sec length). If the temperature is changed immediately after the flash, photoreactivation is not affected.

The results of these experiments can be understood on the basis of the following model. The probability for a photoreactivable particle to be reactivated is proportional to the time integral of the concentration of a photoproduct  $N^*$ . Production and destruction of  $N^*$  are determined by the following reactions.



The first two reactions establish an equilibrium between two bacterial components  $M$  and  $N$  which may or may not be combined with the infecting phage; they are both temperature dependent. The third reaction is the photochemical reaction, the fourth is the reaction by which  $N^*$  is destroyed and eliminated, and the fifth is the reaction of  $N^*$  with phage, which constitutes photoreactivation.

After a long dark period,  $N$  has an equilibrium concentration

$$[N] = \frac{k_1[M]}{k_2}, \quad (12-1)$$

and  $N^*$  has concentration zero.

After a long period of continuous light,  $N$  has an equilibrium concentration

$$[N] = [M] \frac{k_1}{k_2 + k_3 I}; \quad (12-2)$$

and  $N^*$ ,

$$[N^*] = \frac{k_3 I}{k_2 + k_3 I} \frac{k_1 [M]}{k_4 + k_5}, \quad (12-3)$$

which satisfies the hyperbolic relation found experimentally.

After a long dark period, N is present at maximum concentration, given by Eq. (12-1); if a very intense light is turned on, N will be very rapidly and almost entirely transformed into N\*, which consequently will reach a high concentration; during further illumination at the same intensity the concentration of N will remain at a lower steady level [given by Eq. (12-2)] and production of N\* will continue at a constant rate, lower than the initial one. The photoreactivation curve is, therefore, divided into two parts: a very short initial part, whose slope is proportional to  $k_3I$ , and therefore *not* saturated at high light intensity. It measures the velocity of the light reaction; a second longer part, whose slope is proportional to  $k_3I/(k_2 + k_3I)$ . This shows saturation at high light intensities and measures the competition between the dark reaction (12-2) and the light reaction.

The regeneration of N after a flash of very-high-intensity reactions (12-1) and (12-2) can be studied by exposing the infected bacteria to a series of light flashes of equal length and intensity, separated by dark intervals of various length. According to the model, regeneration of N in darkness should follow the relation

$$[N] = \frac{k_1[M]}{k_2} (1 - e^{-k_2t})$$

where time  $t$  is measured from the end of the flash. The experimental data fit this relation very satisfactorily, and determine for reaction (12-2) a time constant of 35 sec at 37°C. Bowen has also studied the effect of temperature on reaction (12-2) by determining  $k_2$  with three independent methods. The results obtained here are not easily interpretable, because the slope of the Arrhenius plot, as determined with two methods, is not constant; for temperatures above 20°C the slope corresponds to an activation energy of approximately 9000 cal/mole, and below 20°C to an activation energy of approximately 17,000 cal/mole.

These experiments have therefore proved that both a light and a dark reaction are involved in photoreactivation, have established their sequence, and have measured the time constant of the reverse dark reaction from N to M.

### 3-6. KINETICS OF PHOTOREACTIVATION OF PHAGE T3

Phage T3 represents the opposite extreme to phage T2 in the T series of phages. It belongs to the smallest group, and has a very short tail. Its radiobiology is in most respects much simpler than that of the larger phages (Dulbecco, unpublished). Its resistance to ultraviolet is about eight times higher than that of free T2, i.e., it is about as high as that found for T2 several minutes after infection (Luria and Latarjet, 1947). Possibly it lacks a special structure which in T2 is more sensitive to ultra-



violet and is therefore responsible for the phenomena of multiplicity reactivation. The photoreactivation curve of T3 has a multiple-hit character, with the number of hits proportional to the ultraviolet dose. The number of active particles  $S$  after a time  $t$  follows strictly the equation

$$S = Be^{-ne^{-ft}},$$

where  $B$  is the total number of photoreactivable particles,  $n$  the average number of photoreactivable hits per phage particle, and  $f$  the photoreactivation rate. Under the assumption that both inactivating and photoreactivating events are distributed at random in the phage population, this relation shows that a photoreactivable particle of T3 requires, to be reactivated, the occurrence of a number of effective events equal to the number of inactivating events.

### 3-7. KINETICS OF PHOTOREACTIVATION OF PHAGE T2 IN CONDITION OF MULTIPLE INFECTION

A bacterium infected with more than one particle of phage T2 inactivated with ultraviolet has a much higher probability of yielding active phage than if infected with one particle only (Luria, 1947; Luria and Dulbecco, 1949). The photoreactivation curve for such multiple-infected bacteria (multicomplexes) is similar to that observed in T3, but does not follow strictly the equation realized in T3, although it tends to it for increasing multiplicity and increasing ultraviolet dose (Dulbecco, unpublished). These findings are taken as indication that phage T2 upon ultraviolet irradiation receives damages in two different structures, of which one, more sensitive to ultraviolet, is photoreactivated by one photoreactivating event; the other, less sensitive to ultraviolet, is photoreactivated like T3. In single infection the damage produced in the more sensitive structure is predominating; in multiple infection the phage can partly dispense with the sensitive part, the more completely so the higher the multiplicity, so that the ultraviolet damage in the other structure becomes predominant.

### 3-8. ACTION SPECTRUM OF THE PHOTOREACTIVATING LIGHT

Bacteriophage T2 adsorbed on *E. coli* B is an excellent material for the determination of the action spectrum of the reactivating light, because the rate of photoreactivation, experimentally determinable, is proportional to the concentration of the immediate photoproducts of  $N^*$  [Sect. 3-5d, Eq. (12-3)]. If the intensity of the photoreactivating light is sufficiently low, we can write: Photoreactivation rate =  $k_3IC$ , where  $C = k_1[M]/(k_4 + k_5)$ , a constant at constant temperature for a given bacterial population.

If the light intensity is measured in quanta per unit time,  $k_3$  is propor-

tional to the product of the absorption coefficient of the photosensitive pigment by the quantum yield.

The determination of the photoreactivation rate and light intensity yields, therefore, the relative values of the absorption coefficient of the pigment, provided the quantum yield is constant at all wave lengths. On this basis a series of points in an action spectrum for phage T2 in single infection have been obtained, by measuring the intensity required for each wave length to produce a standard low rate of photoreactivation, far from the saturation point (Sect. 3-5b). The light used was reasonably monochromatic, having been obtained by isolating lines of the mercury spectrum by glass and liquid filters. The action spectrum is constituted by a single band, extending from about 3100 up to nearly 4800 Å, with a maximum between 3600 and 4000 Å (Fig. 12-5). The unirradiated phage does not absorb light in this region; the ultraviolet-irradiated phage has an absorption band between 3100 and 3700 Å, with a peak at 3300 Å. It is very doubtful that light absorption in this band may be responsible for photo-reactivation; more likely the effective light is not absorbed in the phage.

### 3-9. ACTION OF CHEMICAL SUBSTANCES ON PHOTOREACTIVATION

Reactivation of irradiated bacteriophages has not been obtained with chemical means, either in the presence or in the absence of light. Extracts obtained from sensitive bacteria cannot replace the bacteria in photoreactivation; samples of ultraviolet-treated phages kept in darkness or in light in the presence of yeast extract, or catalase, are not reactivated. Photoreactivation occurs equally well in the presence and in the absence of oxygen; 0.01 *M* cyanide does not affect the rate of the process.

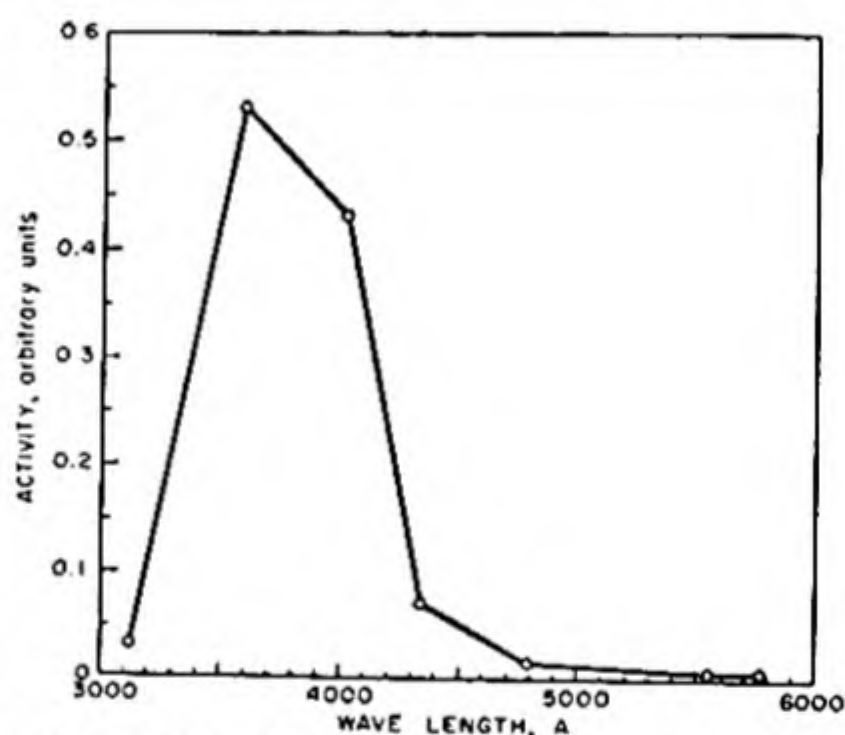


FIG. 12-5. The action spectrum of photoreactivation of phage T2. The activity of each wave length, proportional to the reciprocal of the dose of light expressed in ergs per unit area required for obtaining a standard amount of photoreactivation, is plotted versus wave length. The phage was irradiated with the germicidal lamp, adsorbed on bacteria in buffer, and illuminated in liquid at 37°C.

### 3-10. PHOTOREACTIVATION OF BACTERIOPHAGES INACTIVATED BY AGENTS OTHER THAN ULTRAVIOLET

A small amount of photoreactivation has been found in phages irradiated with X rays (Dulbecco, 1950; Watson, 1950).



### 3-11. PHOTOREACTIVATION OF THE INDUCTION PROCESS OF PHAGE CARRIED LYSOGENICALLY

As discovered by Lwoff *et al.* (1950) cells of *Bacillus megatherium* carrying lysogenic phages can be induced to lyse by ultraviolet irradiation. This effect of ultraviolet can also be photoreactivated, i.e., the carrying bacteria will not liberate the phage if exposed to the photoreactivating light after the inducing exposure to ultraviolet (Jacob, 1950).

Latarjet (1951b) has shown that induction of the same organism is also produced by X rays, and has announced (1951a, b) that the inducing activity of X rays could be counteracted by photoreactivation; this result is surprising since in all other cases X-ray damage has been found to be at most very slightly photoreactivable.

There is a reason to suspect that the apparent reversal of X-ray induction may not be photoreactivation. It has been found that *E. coli* B cells irradiated with a dose of the photoreactivating light which does not kill the bacteria and then infected with active phage T2 lose the ability to produce phage. The effect observed by Latarjet (and possibly also by Jacob) might be similar, and might consist in the loss of the ability of the induced phage to grow in the illuminated bacteria rather than in a photo-reversal of the induction (Dulbecco and Weigle, 1952).

### 4. PHOTOREACTIVATION OF PLANT VIRUSES

Bawden and Kleczkowski (1952) observed photoreactivation of tobacco necrosis virus in French bean and of tomato bushy stunt virus in *Nicotiana glutinosa*. As with bacteriophages, the effect was obtained only when the ultraviolet-irradiated virus was inoculated into the leaves, which were in turn illuminated with white light. A virus preparation whose activity was reduced to about 1 per cent could be restored by the light to 4–10 per cent. No effect was observed in tobacco mosaic virus inoculated into leaves of *N. glutinosa*.

The different photoreactivability of these three viruses may suggest a correlation with their relative content in nucleic acid, which in tobacco mosaic virus is about one-third of that present in the other two viruses (see also Sect. 3-4).

### 5. PHOTOREACTIVATION OF BACTERIA

Extensive observations (Kelner, 1949b, c; 1950a; Novick and Szilard, 1949) have been performed on photoreactivation of bacteria, attention having been focused almost exclusively on the bacterium *E. coli* B/r (Witkin, 1946); a few observations have been made on the strains B and K12 of the same species.

### 5-1. CONDITIONS UNDER WHICH PHOTOREACTIVATION OCCURS

Photoreactivation occurs when the bacteria are suspended in a liquid medium or when they are plated on a solid medium. For quantitative work the first method has been used exclusively. Kelner used bacteria grown for 48 hr with aeration in a glucose-ammonium chloride medium, and then diluted with saline by a factor of 2; Novick and Szilard used resting cells obtained from cultures grown in a lactate-ammonium phosphate medium to approximately  $10^8$  cells/ml, transferred into saline, and incubated in this medium under continuous aeration for 14–18 hr to completely exhaust utilizable reserves; these bacteria were kept in the icebox at 6°C and could be successfully used with reproducible results for about one week.

### 5-2. EFFECT OF THE GROWTH STAGE OF BACTERIA ON PHOTOREACTIVATION

The characteristics of photoreactivation of growing and resting cells of *E. coli* B are very different. This point will be discussed later.

Particularly important for the photoreactivability is the growth condition of the bacteria *after* they have been irradiated with ultraviolet. Kelner has found that if the bacteria, prepared as previously specified, are chilled immediately after irradiation, they can be kept chilled for at least 8 hr without significant effect on subsequent photoreactivation. On the contrary, if they are kept at 37°C after irradiation, their photoreactivability decreases, the decrease being very strong if the bacteria are in a nutrient medium (a decrease by a factor of 1000 in 2 hr); a considerable decrease occurs also in saline, although at a lower rate (Kelner, 1949c; Novick and Szilard, 1949).

### 5-3. COMPARISON OF SURVIVAL CURVES AFTER ULTRAVIOLET TREATMENT IN PRESENCE AND ABSENCE OF PHOTOREACTIVATION

Kelner (1949c) and, independently, Novick and Szilard (1949) have established that these two curves have a simple relation: the ratio between the ultraviolet dose required for obtaining a given survival in the dark and after maximum photoreactivation is constant for any survival. Formally, this phenomenon can be described by saying that the effect of the light is to reduce by a constant factor the ultraviolet dose given to the sample; hence the name of "constant ultraviolet dose reduction" is applied to the observed relation. The demonstration of the principle can be obtained in two different ways (Novick and Szilard, 1949). The first method is to plot the dose required for obtaining a given survival after photoreactivation ( $L$ ) versus the dose observed at the same survival in darkness ( $D$ ); the slope of this line measures the fraction to which the ultraviolet dose is



apparently reduced. The second method is to draw tangents to the two survival curves, in darkness and after photoreactivation, at points of same survival; the pair of tangents drawn to the two curves for any value of the survival must intersect on the ordinate. Novick and Szilard have shown that both conditions are fulfilled in their experiments with *E. coli* B/r (Fig. 12-6).

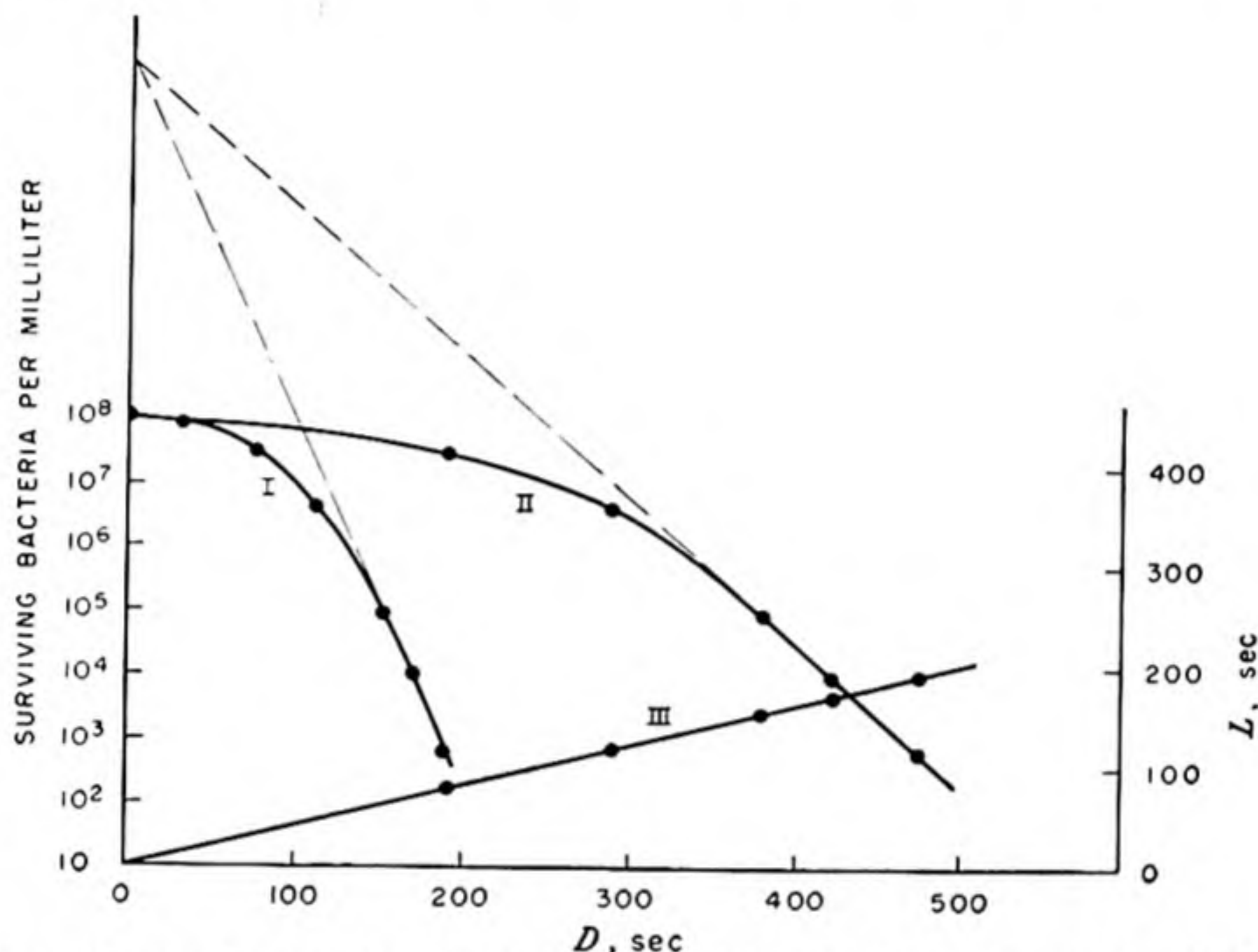


FIG. 12-6. Survival curves for *Escherichia coli* B/r in darkness (curve I) and after maximum photoreactivation (curve II). Curve III gives the ultraviolet dose corresponding to a given survival after photoreactivation versus the dose corresponding to the same survival in darkness. The dotted lines are a pair of tangents to the survival curves at two points of same survival (see text). For survival curves, the logarithm of surviving bacteria (left-hand scale) is plotted versus the ultraviolet dose in seconds of exposure (bottom scale). For the lower curve, the light-ultraviolet dose in seconds of exposure (right-hand scale) is plotted versus the dark-ultraviolet dose (bottom scale). (From Novick and Szilard, 1949.)

In *E. coli* B the relation between the survival in the dark and after photoreactivation depends on the physiological conditions of the bacteria (Novick, personal communication). In *resting* bacteria, obtained with the method previously described, multiple-hit type survival curves are observed both in darkness and after maximum photoreactivation; these curves show constant dose reduction by the light. The survival curve of bacteria from a *growing* culture shows a first period of rapid inactivation, the slope of the curve then decreasing; after maximum photoreactivation a curve of multiple-hit type is obtained. Novick suggests that in growing bacteria the survival curve in darkness is the result of two types of inactivation, of which the first, affecting all but a small fraction of the popula-

tion, is completely reactivated by the light; the second is similar to that observed in resting cells, and shows the constant ultraviolet dose reduction by light.

Novick and Szilard interpret the constant dose-reduction phenomenon in the following way. They assume that inactivation by ultraviolet is due to formation of a poisonous chemical compound in the cells, produced in amount proportional to the ultraviolet dose. The poison is produced in two forms; one sensitive, the other insensitive to light, the ratio between the amounts of the two forms in individual bacteria being independent of the ultraviolet dose. The fraction of survivors after a given ultraviolet dose is determined by the amount of poison of both types present in the cells at the time they are incubated with nutrient medium and permitted to multiply. If the bacterium is exposed to light before this moment, the photosensitive poison is destroyed, and only the photo-insensitive one remains. The formal reduction of the ultraviolet dose is due to reduction in the amount of poison.

This theory can explain well the observed relations of the survival curves in *E. coli* B/r and many other features of photoreactivation in this organism (see Sect. 5-4).

In those microorganisms, in which the survival curve approaches the one-hit type, as in bacteriophages, the amount of the poison able to produce inactivation in the majority of the organisms reduces to a small number of molecules per organism, so that the poison theory of inactivation becomes a "hit" theory.

#### 5-4. KINETICS

The kinetics of photoreactivation of *E. coli* B/r has been worked out by Novick and Szilard (1949) as a development of the poison theory. It is assumed that the photosensitive poison is destroyed by the light in a first-order reaction. As a result, the curve of  $\log [1 - p(t)/p(\infty)]$  versus time (see Sect. 3-5) has a multiple-hit character; this theory could not therefore explain a type of photoreactivation like that found in phage T2, in which this curve is a straight line. In *E. coli* B/r the expectation of the theory is fulfilled. The action of the light in the first minutes of illumination is somewhat less than expected on the basis of the theory, so that a latent period of several minutes is assumed.

The rate of destruction of the poison, calculated according to the theory, is independent of the ultraviolet dose. The calculated rate depends on the intensity of the reactivating light and increases with its intensity but without strict proportionality. The latent period increases when the light intensity decreases.

If photoreactivation is carried out with a dose which does not give maximum photoreactivation, the survival curves still follow the constant dose reduction principle.



Novick (personal communication) has checked the poison theory with the following experiment: A sample of *E. coli* B/r was irradiated with a dose  $D$  of ultraviolet and then photoreactivated. After this operation the survival corresponded to a dose  $L_1$  ( $L_1 < D$ ) of ultraviolet given in the dark. According to the poison theory, there should have been no difference between these photoreactivated bacteria and bacteria which received originally a dose  $L_1$  of ultraviolet and were photoreactivated. If now a new ultraviolet dose is given to the photoreactivated bacteria, their survival curve should be equal to the original survival curve in absence of photoreactivation, starting at the ultraviolet dose  $L_1$ . This was actually found. After a second stage of photoreactivation the survival of the bacteria became equivalent to a dark ultraviolet dose  $L_2$ ; the twice-photoreactivated sample was exposed to ultraviolet for the third time, and again the survival curve was similar to the original one, starting at a dose  $L_2$ .

#### 5-5. ACTION SPECTRUM OF THE PHOTOREACTIVATING LIGHT

Preliminary results on the shape of the action spectrum for bacterial photoreactivation have been published by Kelner (1950b), who has described in *E. coli* a peak near 4000 Å, and by Knowles and Taylor (1950), who determined that the greatest effect occurs for wave lengths between 3500 and 4500 Å, with a maximum at 3600 Å. A more extensive determination of the action spectra of *E. coli* B/r and *Streptomyces griseus* spores has been published by Kelner (1951). The relative efficiency of lights of different wave lengths was determined by comparing the amount of light energy required to produce a standard "degree of photoreactivation" (the fraction of inactivated cells that have been photoreactivated) after a standard ultraviolet irradiation. For values of the degree of photoreactivation near the standard value, the degree of photoreactivation is a linear function of the logarithm of the light energy, and interpolation of the data is possible. In the range of light intensities used, the reciprocity law was found to hold for exposures varying between 1.6 and 75 min for *S. griseus* and between 5 and 52 min for *E. coli*. However, long exposures should be relatively less effective in *E. coli* owing to the decay of photoreactivability with the time of sojourn of this organism at 37°C in saline (Kelner, 1949c; Novick and Szilard, 1949); this decay might be masked by saturation occurring at high light intensity. This may affect somewhat the shape of the action spectrum.

The spectral region examined consisted partly of lines of the mercury spectrum isolated by glass filters and partly of bands separated out of the continuous spectrum of an incandescent bulb by interference filters. The spectral regions of the second type are not well defined because the interference filters do not cut off very sharply. This introduces some uncertainty for the points so determined.

The action spectrum for *E. coli* (Fig. 12-7) is similar to that of photoreactivation of phage T2 adsorbed on the same bacterium (see Sect. 3-8), the peak being more accurately determined at 3750 Å. An unexpected result is the finding that the action spectrum for *S. griseus* spores extends considerably farther into the longer wave lengths, the peak being at 4360 Å. The difference between the two spectra is undoubtedly substantial; possible artifacts are discussed by the author, who also discusses the possibility that the photosensitive pigment of *S. griseus* spores is a porphyrin.

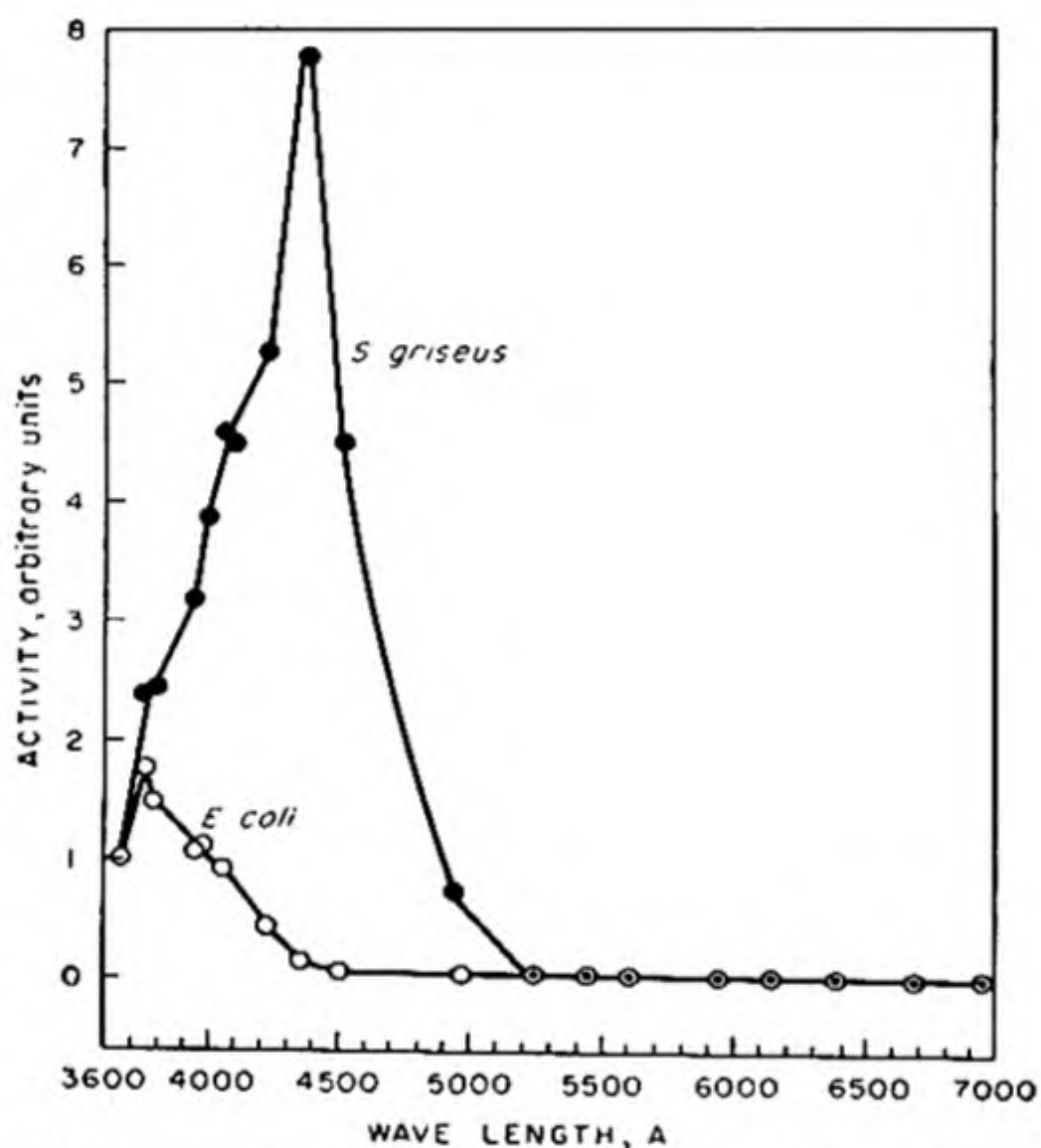


FIG. 12-7. The action spectra of photoreactivation of *Streptomyces griseus* and of *Escherichia coli* B/r. The activity of each wave length, proportional to the reciprocal of the dose of light, expressed in quanta per unit area, required for obtaining a standard degree of photoreactivation is plotted versus wave length. (From Kelner, 1951.)

#### 5-6. CHEMICAL ACTIONS CONNECTED WITH PHOTOREACTIVATION

Monod *et al.* (1949) have observed that bacteria of the strain *E. coli* K12 irradiated with ultraviolet in citrate buffer and plated on a synthetic agar medium are not photoreactivable, and that photoreactivability appears, together with a considerable dark reactivation, if catalase or ferrous sulfate is added to the medium. Latarjet and Caldas (1952) showed that the phenomenon is rather erratic and presumably finely dependent on the physiological state of the bacteria. They found that catalase restoration can take place occasionally in the absence of light, but usually requires illumination; that it is absent in other strains of the same species, *E. coli* B



and B/r; that it is absent after X-ray irradiation; and that it takes place in *B. megatherium* 899, which, like *E. coli* K12 is lysogenic, whereas *E. coli* B and B/r are not so. Therefore some relation with lysogenicity is indicated.

The catalase effect seems to be an important feature; it is probably not identical with photoreactivation but related to it.

Johnson *et al.* (1950) have found that oxygen is not required for bacterial photoreactivation.

#### 5-7. ACTION OF PHOTOREACTIVATION ON THE INDUCTION OF MUTATION BY ULTRAVIOLET IN BACTERIA

This problem is of great importance for understanding the relation between inactivation and induction of mutations. Observations have been carried out mainly on the induction of the mutation to resistance to bacteriophages in *E. coli* B/r (Kelner, 1949b, c; 1950a; Novick and Szilard, 1949). Two different techniques have been used for determining the extent of the mutagenic action and the effect of photoreactivation. Kelner has determined the number of *mutations* present under various experimental conditions according to the spray technique of Demerec (1946). Novick and Szilard have determined the number of *mutants* present in a culture arising from a treated bacterial suspension after the bacteria in the sample have been allowed to undergo on the average about ten divisions. With regard to this method, it must be observed that bacteria treated with ultraviolet show a variable lag period before starting to divide, and that mutations induced by ultraviolet become phenotypic after an additional lag (Newcombe and Scott, 1949); the fraction of mutants in the population after growth might therefore be affected by variations in the lag values.

Kelner studied the behavior of zero-point mutations (mutations arising before any division of the ultraviolet-treated cells has taken place) and of delayed mutations (manifested after several cell generations). The zero-point mutations appear to be completely suppressed if the bacteria after the ultraviolet treatment are exposed to the photoreactivating light; the fraction of delayed mutations, on the contrary, is little or not at all affected by the light.

Novick and Szilard found that the fraction of mutants in the total population is considerably decreased by the action of the light, and that the fraction of mutants observable after photoreactivation is comparable with the amount obtainable with a lower ultraviolet dose in the absence of photoreactivation (Fig. 12-8). Induction of mutations by ultraviolet would therefore be affected by photoreactivation in a similar way as the reactivation, with a constant ultraviolet dose reduction produced by light.

The difference in the results obtained by Kelner and by Novick and Szilard cannot be accounted for by the difference in the method used, since

Kelner's data show that the effect of photoreactivation on the delay in appearance of mutations is, if any, a reduction of the lag period. This would affect the fraction of mutants in the method used by Novick and Szilard in the direction of increasing it—in a direction, therefore, which is opposite to the observed difference.

Kelner points out that the curve giving the frequency of delayed mutations among survivors as a function of ultraviolet dose for *E. coli* B/r rises

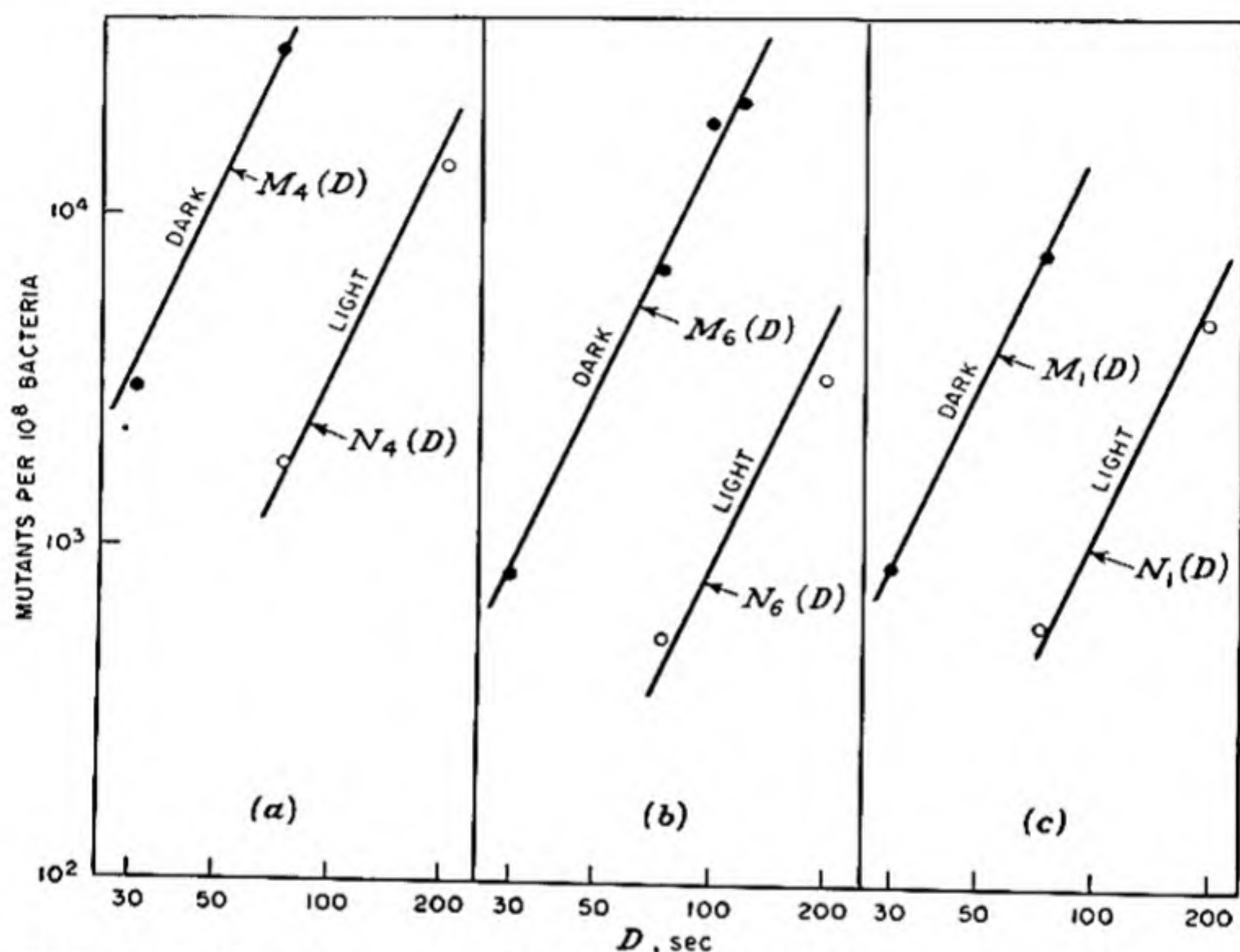


FIG. 12-8. Logarithm of number of phage-resistant mutants per  $10^8$  bacteria as a function of the logarithm of the ultraviolet dose  $D$  in darkness and after maximum photoreactivation for (a) phage T4, (b) phage T6, and (c), phage T1. The bacteria had passed through 10 generations in liquid culture prior to being assayed for the mutants. (From Novick and Szilard, 1949.)

sharply at low ultraviolet doses and less at high ultraviolet doses (Demerec and Latarjet, 1946); therefore, if the light causes a constant reduction of the ultraviolet dose also for the mutagenic effect, the relation between the fraction of mutants before and after photoreactivation should vary according to the ultraviolet dose used. At a low ultraviolet dose, where the curve giving the fraction of mutations versus the ultraviolet dose has a steep slope, the fraction of mutants should considerably decrease after photoreactivation, whereas at a high dose, where the slope is less, the fraction of mutants should be less affected. This can account for part of the difference in the results obtained by Kelner and by Novick and Szilard,



since the latter authors used lower doses in their experiments. This explanation does not account for all the differences, however, since the fractions of mutants among survivors in points corresponding to the survival values observed by Kelner in the absence and in the presence of photoreactivation differ, according to Demerec and Latarjet (1946), by a factor of 10 or more, less mutations being present at the lower dose.

The effect of photoreactivation on the so-called "zero-point mutations" seems not sufficiently documented in view of the fact that detection of these mutations involves infection with phage of a very large number of bacteria inactivated with ultraviolet but able to adsorb the phage. Under these conditions the multiplicity of phage infection may become too low, so that a considerable fraction of bacteria may divide before infection.

Newcombe (1950) and Newcombe and Whitehead (1950) have observed that the mutagenic effect of ultraviolet on *E. coli* B/r (streptomycin-resistance mutants and color-response mutants on mannitol-tetrazolium agar) is particularly strong at low doses of ultraviolet. At these low doses the reversal of the mutagenic effect by photoreactivation is also strong. At a dose of 500 ergs/mm<sup>2</sup> more than 90 per cent of the potential mutants fail to appear after photoreactivation. At larger doses the mutations produced by ultraviolet alone do not increase with dose, and their photoreactivability tends to zero.

The results show that the effect of photoreactivation on these mutations can be approximately described on the basis of the dose-reduction principle, as in the case of Novick and Szilard (1949), although the mutagenic effect of ultraviolet is reduced by photoreactivation to a greater extent than the killing action as determined by Kelner (1949c). This difference may not be significant since the amount of reduction of the killing action has not been determined under the same conditions.

## 6. PHOTOREACTIVATION IN *STREPTOMYCES* AND FUNGI

Photoreactivation was first discovered in spores of *Streptomyces griseus* irradiated with ultraviolet (Kelner, 1949a). Spores suspended in saline or distilled water are exposed to ultraviolet and then treated with visible light with the following results: (1) the ratio of the number of active spores after photoreactivation to the number of active spores before photoreactivation increases with the ultraviolet dose; (2) with light of constant intensity the number of active spores tends to a maximum value with time of illumination; (3) increasing the intensity of the reactivating light increases proportionately the rapidity of recovery within certain limits; and (4) the rapidity of recovery increases with the temperature existing during light treatment, up to temperatures of 50°C.

The action spectrum of photoreactivation of *S. griseus* spores (Fig. 12-7)

has been determined in detail by Kelner (1951); it has already been discussed (Sect. 5-5) together with the bacterial action spectrum.

In *Neurospora crassa*, Goodgal (1950) has observed that the survival of microconidia irradiated with ultraviolet is affected by photoreactivation, which produces an increase in the number of active conidia with a constant reduction in ultraviolet dose. The frequency of morphological mutants among survivors has also been studied and found to be similarly reduced by photoreactivation. The ultraviolet dose reduction is of the same order of magnitude for lethal and mutagenic action, since a given survival corresponds to a constant fraction of mutants, independently of the presence or absence of photoreactivation. This result is therefore similar to that obtained by Novick and Szilard (1949) in *E. coli* (Sect. 5-7). Goodgal attributes these results to one of two possibilities: (1) killing of microconidia by ultraviolet is due to a lethal mutation, and light decreases killing by hindering the occurrence of mutations; or (2) killing and mutations are produced by a common mediator formed under ultraviolet treatment and destroyed by the light.

Brown (1951) studied the effect of photoreactivation on the reversion of the inositol requirement in *N. crassa* by irradiating mononucleate microconidia with ultraviolet, and found that the fraction of mutated conidia among the survivors is considerably less after photoreactivation. This result cannot be due to lack of photoreactivation of inactive mutants, since after photoreactivation the absolute number of mutants increases.

## 7. PHOTOREACTIVATION IN YEAST

Photoreactivation in yeast has been demonstrated by Kelner (1949a) and by Swenson and Giese (1950). The latter authors have shown that the mechanism of enzymatic adaptation to galactose fermentation in *Saccharomyces cerevisiae* is damaged by ultraviolet and that this effect also can be partly undone by exposing the cells irradiated with ultraviolet to a strong white light. Several points in an action spectrum for the ultraviolet destruction of adaptability have given results compatible with the assumption that the radiation acts on nucleic acid (Swenson, 1950).

## 8. PHOTOREACTIVATION IN PROTOZOA

Kimball (1949) and Kimball and Gaither (1950, 1951) have studied photoreactivation in *Paramecium aurelia* by using monochromatic radiation of wave length 2804 and 2650 Å in the majority of the experiments. In this organism irradiation with ultraviolet has a number of effects: retardation in cell division, killing of a fraction of animals before autogamy, reduced vigor after autogamy, and morphological changes in the structure of the macronucleus.



The delay in the onset of cell division is put on a quantitative basis by determining the time at which the sixth division takes place; after this division no further delay occurs. When the time of the sixth division is plotted against the ultraviolet dose, for each wave length used two different curves are obtained—one in darkness, the other after photoreactivation (Fig. 12-9). The experimental points have high dispersion, but curves drawn among the points show that the effect of photoreactivation is compatible with a constant ultraviolet dose reduction.

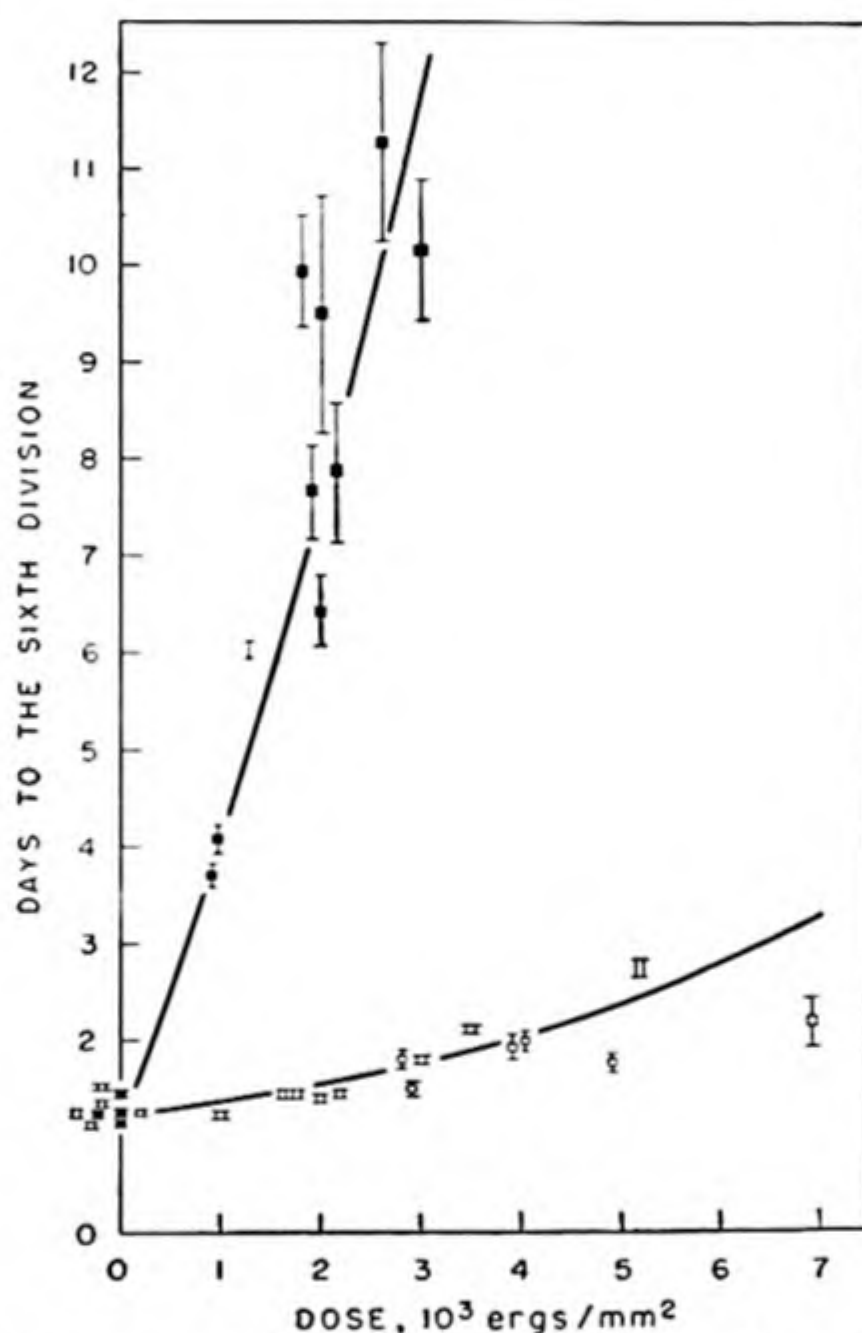


FIG. 12-9. Days to the sixth division plotted against dose of 2804 Å ultraviolet. Curve I, dark; curve II, light. Each point represents the arithmetical mean for 5 to 54 lines of descent from a single experiment. (From Kimball and Gaither, 1951.)

Death before autogamy behaves in a similar way as the retardation of cell division, and the authors consider that the two phenomena are likely interdependent.

Reduced vigor after autogamy, on the contrary, is not related to the two effects already described, since it occurs in animals which had completely recovered from previous radiation disease. Since it occurs after homozygosity has been produced, it has very likely to be attributed to gene mutations; however, direct evidence for this has not been presented. Reduced vigor is affected by photoreactivation in a similar way as the genetic effect.

Changes in the structure of the macronucleus after ultraviolet irradiation consist in clumping of granular structures with subsequent vacuolization (Kimball, 1949). If the animals are exposed to the photoreactivating light following ultraviolet irradiation, some clumping occurs, but fusion and vacuolization do not ensue; the clumps produced are subsequently dissolved.

## 9. PHOTOREACTIVATION IN ECHINODERM ZYGOTES AND GAMETES

Blum and coworkers have extensively studied the delaying effect of ultraviolet on cleavage in *Arbacia punctulata* eggs, and the effect of photoreactivation on this delay (Blum . . . Robinson, 1949; Blum . . . Loos, 1949; Blum, Loos, and Robinson, 1950). In fertilized eggs irradiated with a Hanovia intermediate pressure arc before cleavage, with filters absorbing most of the visible spectrum, the time of onset of division is delayed, the delay markedly affecting the first and less the second and third division. In eggs exposed to the light of a fluorescent lamp after ultraviolet irradiation, the delay is greatly reduced.

Eggs irradiated with ultraviolet before fertilization show a similar delay in the onset of the first few divisions following fertilization; treatment of the unfertilized eggs with visible light after ultraviolet irradiation decreases the delay. The delay is not affected if the eggs are illuminated with visible light before ultraviolet treatment.

Unfertilized eggs can be separated by centrifugation into two parts, one yellow containing all the echinochrome pigment, the other white containing the nucleus. The white halves are damaged by ultraviolet and photoreactivated like whole eggs. This shows that the echinochrome pigment is not required for this type of photoreactivation. If the enucleated halves are treated with ultraviolet and then fertilized, no delay occurs, suggesting that the site of action of ultraviolet is in the nucleus.

The effective spectral range for photoreactivation of division delay is between 3000 and 5000 Å.

In other experiments the effect of ultraviolet irradiation and photoreactivation on sperm has been studied (Blum, Robinson, and Loos, 1950, 1951). When ultraviolet-treated sperm is used for fertilization of non-irradiated eggs or egg halves (both nucleated and enucleated ones), delay in the time of cleavage occurs. Illumination of the ultraviolet-irradiated sperm with photoreactivating light does not affect the delay, which is, on the contrary, reduced if the eggs fertilized with ultraviolet-irradiated sperm are exposed to the photoreactivating light. The authors point out the similarity of this interesting observation with the situation found in photoreactivation of bacteriophages in which the bacteriophage can be reactivated only if adsorbed on the sensitive bacterium (see Sect. 3-3). No photoreactivation was observed after X-ray treatment.



Similar results have been obtained on the same material by Marshak (1949a, b), who, however, could not detect photoreactivation in unfertilized eggs. This author explored the possibility of some chemical actions in the ultraviolet effect and in photoreactivation by treating gametes and zygotes, before, during, and after irradiation with ultraviolet and visible light, with the following substances: adenine, streptomycin, folic acid, 4-amino-*n*-methyl folic acid (a folic acid antagonist), and riboflavin (on reactivation of zygote only). None had any effect.

Wells and Giese (1950) have investigated the photoreactivation of the cleavage delay subsequent to ultraviolet treatment in gametes and zygotes of the sea urchin *Strongylocentrotus purpuratus*, with experiments similar to those performed by Blum and coworkers. Wells and Giese used for inactivation monochromatic ultraviolet obtained with a quartz monochromator, and compared the photoreactivability of delay produced by several wave lengths between 2450 and 3130 Å. It appears that photoreactivation occurs in eggs treated with ultraviolet of all wave lengths tested with similar efficiency except at the shortest wave lengths, 2450 Å, where photoreactivation is reduced. This wave length has a strong effect on the surface of the eggs, whose membrane is raised. The lower photoreactivability of eggs damaged by treatment with this wave length is attributed to ultraviolet damage to the surface membrane.

Wells and Giese found that sperms are considerably damaged by the photoreactivating light, so that photoreactivation cannot be detected readily. A small amount of photoreactivation could, however, be demonstrated also on the naked sperm, contrary to the findings of Blum, Robinson, and Loos (1950) with *Arbacia*.

The most effective spectral region for photoreactivation in *Strongylocentrotus* gametes and zygotes lies between 3660 and 4300 Å.

#### 10. PHOTOREACTIVATION IN SALAMANDER LARVAE

Blum and Matthews (1952) studied the effect of photoreactivation on the killing effect of ultraviolet radiation on larvae of *Amblystoma maculatum* and *A. opacum*. When the larvae were irradiated with a single massive dose of ultraviolet, no photoreactivation took place; but when the larvae were exposed to repeated low ultraviolet doses, each being followed by exposures to the photoreactivating light, the fraction of surviving larvae was much greater in the light-treated group than in that kept in darkness. It is remarkable that the effect of photoreactivation was still present when the larvae were treated with light 20 hr following ultraviolet exposure; in all other organisms photoreactivability is generally lost within a few hours. The spectral region in which the photoreactivating light was active is similar to that observed in other organisms (3000–5000 Å).

## 11. PHOTOREACTIVATION IN HIGHER PLANTS

Bawden and Kleczowski (1952) found that the first-formed leaves of *Phaseolus vulgaris*, if isolated from the plant and kept in darkness after exposure to ultraviolet light, acquired a bronze color owing to death of the cells of the upper epidermis. If, following ultraviolet treatment, the leaves were illuminated with daylight, the bronze color was not produced and the majority of the cells appeared intact.

## 12. CONCLUSIONS AND SUMMARY

1. Photoreactivation is a very widespread phenomenon, affecting changes of seemingly different nature produced by ultraviolet in organisms of different levels of organization. The fact that most of the known biological effects of ultraviolet irradiation have been photoreactivated seems to suggest that they all have a common step.

2. The effect of photoreactivation is almost completely specific for damages produced by ultraviolet radiation. The susceptibility of damages produced by different ultraviolet wave lengths to photoreactivation is little known, and more complete determinations would be desirable.

3. The site at which the photoreactivable damage is produced is a nucleoprotein, as shown by the results obtained with bacteriophages (Sect. 3-3), plant viruses (Sect. 4), echinoderm sperm (Sect. 9) and yeast (Sect. 7); it is probably nucleic acid, since Hershey and Chase (1952) have shown that at least most of the protein part of the bacteriophage does not enter the bacterium it infects. In this respect there seems to be a correlation between photoreactivability and the nucleic acid content of viruses (Sect. 3-4 and Sect. 4).

4. The mechanism of reactivation requires substances of "cytoplasmic" nature. The action spectra obtained seem to indicate that different pigments are responsible for the absorption of the photoreactivating light in different organisms (Sects. 3-8 and 5-5). This result is surprising in view of the universal nature of the phenomenon. However, the action spectrum of bacteriophage T2, of *E. coli* B/r, and of *Streptomyces griseus* spores could all be due to light absorption in the same substance, for example, in a flavin, provided that the quantum yield varied with the wave length in a different way in different organisms.

5. The type and sequence of reactions involved in photoreactivation are rather clearly understood in bacteriophages (Sect. 3-5), and presumably the same results will be found true for other organisms. The light acts on a pigment in equilibrium with a nonpigment substance; the probability of photoreactivation is proportional to the concentration of the product of the light action on the pigment.

6. The killing effect of ultraviolet is reverted by one quantum of the



photoreactivating light in bacteriophage T2 in single infection (Sect. 3-5), by a small number of light quanta in the same phage in multiple infection (Sect. 3-7), and in phage T3 in single infection (Sect. 3-6); by a larger number of quanta in bacteria (Sect. 4-4). It is possible that at an elementary level of organization photoreactivation is a one-quantum phenomenon, and that higher numbers of quanta are required at higher levels. More extensive kinetic investigations would be desirable in various types of microorganisms.

7. The photoreactivation of the mutagenic effect generally appears as a constant ultraviolet dose reduction (Sect. 5-7 and Sect. 6), indicating the intimate similarity with the photoreactivation of the killing effect. The two effects could be chemically identical but biologically different owing to a different localization of the changes responsible for them.

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## CHAPTER 13

### Sunburn

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" . . . I am black; because the sun has looked upon me."

—Song of Solomon

*Erythema. Suntan. Photosensitization. Protection against sunburn. Mechanisms. Fact and fancy. References.*

Although sunburn must always have been an obvious nuisance to man, it seems to have received little attention from scientists before the present century. To be sure, the true nature of the phenomenon could not have been understood until the discovery of ultraviolet radiation in 1801<sup>3</sup> but it was another half century before it was recognized that sunburn is caused by this agent and not by heat.<sup>4,5</sup> The first recorded statement I have found that sunburn is caused by ultraviolet radiation is in an account by Charcot which appeared in 1858. This describes two cases of burns from electric arcs and cites similar experiences by the physicists Foucault and Despretz. Foucault had found that uranium glass protected against the burning, which was attributed to the ultraviolet or, as they were then called, the "chemical" rays. Charcot clearly recognized that these burns were comparable to sunburn. Widmark in 1889 and 1891 made a more complete study, finding that the rays from a carbon arc passing through

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<sup>3</sup> In that year Ritter found that the sun's spectrum beyond the violet caused the blackening of silver chloride (Ritter, 1803). One year earlier Herschel had shown the existence of the infrared.

<sup>4</sup> Actually, unconcentrated sunlight does not heat the skin enough to produce a burn. For a discussion see Blum (1945).

<sup>5</sup> Historically interesting is a paper by John Davy (1828), who concluded that, in order to elicit a burn, all wave lengths must affect the skin simultaneously.



quartz elicited sunburn, whereas those passing through window glass did not. This would indicate that the sunburn-inducing radiation includes no wave lengths longer than those cut off by window glass, i.e., about  $0.32\ \mu$ , where modern studies show the long-wave-length limit for sunburn to be.<sup>6</sup> Widmark was particularly concerned with sunburn of the eyes, which he showed to be brought about by the same wave lengths as sunburn of the skin. By the turn of the century ultraviolet radiation seems to have been generally recognized as the cause of sunburn (e.g., Hammer, 1891; Finsen, 1900; Möller, 1900), although the spectral region was not delimited more exactly. The first attempt to do this was made by Henri and Moycho in 1914 who used the rabbit as the experimental animal; but the first accurate determinations on human skin were not carried out until the 1920's (Hausser and Vahle, 1922; Hausser, 1928).

Modern studies of sunburn date from about the same time, but, with certain notable exceptions, these have been sporadic in occurrence and inconclusive in character. Perhaps this may be attributed to the complexity of the problem and the difficulties of experimentation in this field, as well as to lack of interest among scientific investigators. In contrast to the relative paucity of experiments on sunburn is the plethora of popular fancies, from which scientists are not altogether immune. A good many false ideas find their explanations in unrecognized physical factors—the optics of the skin or the spectral quality of sunlight; others, in the complex nature of the physiological responses. It is hoped that some misunderstandings may be corrected in the course of this chapter, although a host of questions about sunburn will remain unanswered.

Sunburn involves a number of tissues of the skin, and can be understood only in terms of the anatomy and physiology of that organ. Frequent reference is made in the following pages to layers of the skin which the reader may identify in Fig. 13-1. The character of sunburn may vary to some extent with the severity of exposure and from person to person, but it always presents the same general picture. An hour's exposure to bright midday summer sunlight is usually followed by reddening of the exposed area. This *erythema of sunburn* is the gross manifestation of dilation of the minute vessels of the dermis. The erythema may be accompanied by slight swelling, which becomes more pronounced if the exposure is prolonged. Blistering and desquamation may result from severe exposures, and there may be some pain and itching. The erythema is normally replaced after a few days by *suntan*, the brown color resulting from redistribution and increase in the melanin pigment of the epidermis. The suntan may persist for months or years. Not grossly observable, but of

<sup>6</sup> Bowles (1889), who was unaware of Widmark's experiments, arrived at the conclusion that ultraviolet radiation is responsible for sunburn on the basis of experiences in the Alps. The article is of interest as reflecting the generally vague ideas about radiation current at that time.

major importance with regard to sensitivity to subsequent exposure, is the hyperplasia of the epidermis which results in thickening of both the horny layer (corneum) and the viable malpighian layer. The hyperplasia is preceded by degenerative changes in epidermal cells, and there is migration of leukocytes out of the vessels of the dermis. Considering these changes, sunburn may be classified as an acute inflammatory process, comparable to that associated with superficial burns of any kind. The term "sunburn" is used here to include all these changes without regard to the intensity of the response, whether there is only a fleeting redness or whether severe blisters develop. The term is applied when the sunburn results

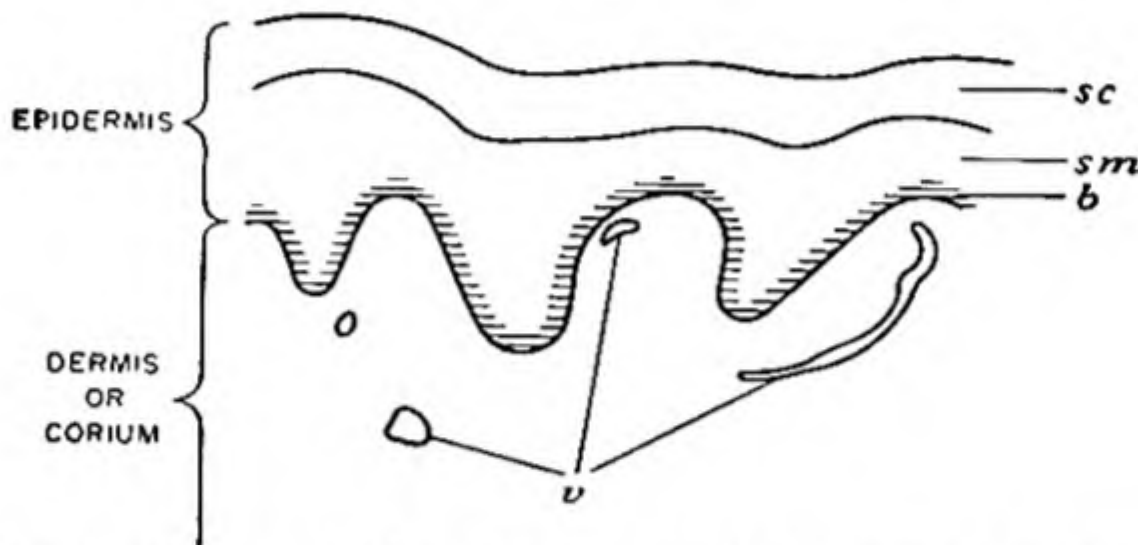


FIG. 13-1. Diagram of cross section of skin from the human shoulder, showing the various layers to which reference will be made in the text. The *epidermis* is the outer layer consisting of: *sc*, the *stratum corneum* or *horny layer*, and *sm*, the *stratum Malpighii*. The cells at the bottom of the malpighian layer constitute the basal cell layer *b*. The "prickle" cells lie superficial to the basal cells. The *dermis* or *corium* includes the tissues of the skin lying beneath the basal cell layer of the epidermis. In the papillary layer of the corium are situated the most superficial blood vessels (*v*). (After Maximov and Bloom, 1940.)

from exposure to ultraviolet radiation from an artificial source as well as when it is caused by natural sunlight.

Various aspects of sunburn stem from the action of ultraviolet radiation of wave lengths shorter than about  $0.32 \mu$  on the cells of the malpighian layer of the epidermis. The effects on the cells are no doubt comparable to those of ultraviolet radiation on living cells in general, but they are manifested here in terms of a complex of secondary processes.<sup>7</sup> These may be treated separately for purposes of study so long as their basic origin and interrelations are not lost sight of. In this chapter various responses which follow the primary photochemical reaction in the cells of the malpighian layer will be considered separately. Later, the mechanism as a whole and the factors influencing sunburn under conditions of natural sunlight will be considered. Sunburn of the eyes also occurs and may be

<sup>7</sup> That ultraviolet radiation may have more than one primary effect on the cell is shown by Blum *et al.* (1954).



very painful and disabling. This subject will not be discussed here, the reader being referred to an earlier review (Blum, 1945).<sup>8</sup>

### ERYTHEMA

The erythema, which is the first grossly observable manifestation of sunburn, appears only on the area exposed to the ultraviolet radiation, being quite sharply delimited from the surrounding normal skin. Unless the dosage is very severe or prolonged, the erythema does not appear immediately; with moderate doses there is usually an interval of an hour or more. The erythema may persist for a few days, fading imperceptibly into the brown color of suntan. Histological examination shows no changes in any layer before the gross appearance of reddening.<sup>9</sup> The erythema itself is revealed as the enlargement and engorgement of the minute vessels of the dermis (corium), which lie just below the epidermis (see Fig. 13-1). The red color results from the increased amount of blood in these vessels. Intracellular edema and the migration of leukocytes into the surrounding tissues begin at about the same time as the erythema.

Although these first detectable changes involve the vessels of the dermis, this is not the site of the photochemical reaction that initiates them. Only a very small fraction of the incident ultraviolet—virtually restricted to the longer wave lengths—ever reaches the most superficial vessels (see Fig. 13-7), yet shorter wave lengths, e.g.,  $0.28\ \mu$ , which are completely absorbed in the epidermis, do elicit erythema.<sup>10</sup> Ultraviolet radiation that is absorbed in the epidermis must, then, cause changes there that lead in some way to dilation of the vessels in the layer beneath. It seems necessary to conclude that photochemical changes in the epidermis lead directly or indirectly to the elaboration of some mediating substance or substances which move down to the superficial vessels of the dermis and cause their dilation. The time between dosage with ultraviolet and appearance of erythema is presumably consumed in the elaboration of these dilator substances and their penetration into the dermis. The horny layer of the epidermis, the corneum, is a nonliving structure built from the viable cells of the malpighian layer beneath it. There is a transition zone between the two layers where it may be difficult to distinguish

<sup>8</sup> It has been claimed that ultraviolet radiation raises the threshold of scotopic vision, but this seems to have been definitely ruled out by recent studies by Wald (1952) who shows conclusively that radiation which might damage the retina does not reach it.

<sup>9</sup> The histological studies of Keller (1924a, b), Miescher (1930), and Hamperl *et al.* (1939a) may be cited as the most complete. There are points of apparent divergence which probably result from differences in the spectral quality and dosage of radiation and the times at which the biopsies were made, but the picture is similar in all three studies.

<sup>10</sup> Evidence that the longer wave lengths that penetrate slightly to the dermis may have a direct effect there will be presented a little later on.

whether cells are living or dead. The corneum acts as a filter, preventing a large proportion of the incident ultraviolet radiation from reaching the malpighian, and until very recently it was thought to take no active part in the sunburn complex. Rottier (1952, 1953) and Rottier and Mullink (1952) have now presented strong evidence, however, that photochemical changes in the corneum may contribute erythema-producing substances.

*The Erythematous Threshold.* The most obvious criterion for quantitative studies of sunburn is the appearance of erythema. The erythematous threshold, i.e., the amount of ultraviolet radiation required to produce just perceptible reddening, has been quite generally used, and is up to now the only feasible measure available. As will be seen, however, it is not a very accurate one and should be interpreted with caution. The erythematous threshold has been measured in various ways, but all involve more or less the same type of operation. Usually the skin is covered with a template having a series of small holes through which the ultraviolet radiation may reach the skin. A different dose is applied through each of these holes. The threshold is usually determined after some arbitrary period, say, 24 hr. At this time, if the doses have been chosen appropriately, the highest ones will be represented by red areas corresponding to the positions of the holes in the template. The intensity of the redness will fall off with the dose, those areas having received the lowest doses showing no erythema whatsoever. The dose which has elicited a just perceptible erythema, or the average between this and the next lower one which has produced no erythema, is usually chosen as the threshold. The exact value for the threshold will depend, of course, on the time elapsed between the exposure to ultraviolet radiation and the time of observing, since the erythema builds up rather slowly and then falls off. Studies of the rate of increase and decline of erythema have shown that these factors may vary widely in different individuals (e.g., Schall and Alius, 1926).

There are obvious sources of error in determining the erythematous threshold. The presence or absence of a very slight reddening is a difficult end point and is subject to considerable uncertainty in reading since the degree of flushing or tanning of the skin affect the contrast. On the whole, photography has not helped to standardize such measurements, the unaided eye being more sensitive and accurate. It is probable, in any case, that inherent variability in the erythematous response is greater than the errors in reading.<sup>11</sup> The threshold not only varies widely among individuals but also from one area to another. For example, the palm of the hand or the sole of the foot can hardly be sunburned. On the other hand, the skin of the torso is rather uniformly sensitive. The threshold is affected by the amount of previous exposure to ultraviolet radiation. It may vary widely from one observation to another without known cause

<sup>11</sup> For a discussion of errors of measurement of the erythematous threshold, see Blum and Terus (1946b).



(Blum and Terus, 1946b), and there are regular changes with season and other factors (see Ellinger, 1941). All these factors must be taken into account in quantitative studies, but there are more serious difficulties in the interpretation of measurements which will need discussion.

*The Erythematous Spectrum.* The value of action spectra in characterizing photobiological responses—aiding in favorable cases in detecting the light-absorbing substance in the primary photochemical process—has been discussed elsewhere in this book (see also Blum, 1950). The action spectrum for sunburn as measured in terms of the erythematous threshold is generally spoken of as the “erythematous spectrum.” This has been determined by several workers whose results are in close agreement as far as the general character of the spectrum is concerned (Hausser, 1928; Luckiesh *et al.*, 1930; Coblentz *et al.*, 1932). All these investigators found a maximum at about  $0.295 \mu$  and a minimum at about  $0.28 \mu$ . It is customary to plot the reciprocal of the threshold against wave length, as in Figs. 13-2, 3, and 7.<sup>12</sup>

The shape of the erythematous spectrum must be affected by the corneum, which overlies the malpighian layer and acts as a semi-opaque filter absorbing the wave lengths of the erythematous spectrum selectively. The corneum is composed of flakelike elements and, owing to the reflection and refraction of the light at the boundaries of these, the incident beam is scattered very effectively.<sup>13</sup> This scattering greatly enhances the attenuation of the radiation and renders measurements of the true absorption difficult.<sup>14</sup> Absorption spectra for human epidermis treated in various ways to reduce scattering are shown in Fig. 13-2. Curves III and IV display the least scattering and may be taken as giving the most reliable picture of the true absorption. Proceeding toward shorter wave lengths, we see that absorption begins to increase rapidly in the neighborhood of  $0.3 \mu$ , reaching a maximum at about  $0.28 \mu$ , then falling to a minimum

<sup>12</sup> Hamperl *et al.* (1939b) state that they have found maxima in the erythematous spectrum at  $0.295 \mu$  and  $0.26 \mu$  with a minimum at  $0.275 \mu$ , and, for some skins with a very thin corneum, another maximum at  $0.23 \mu$ . These findings are described in a brief note with no further detail and I have found no more complete description.

<sup>13</sup> Henschke (1948) has shown that the erythematous threshold for radiation striking the skin at different angles follows the cosine law, which is characteristic of completely diffusing surfaces.

<sup>14</sup> In a layer which both absorbs and scatters, as does the corneum, the attenuation of a monochromatic parallel beam in passing through matter may be expressed as

$$I_l = I \exp [-k(r_\lambda, s_\lambda)l] \quad (13-1)$$

where  $I$  and  $I_l$  represent, respectively, the incident intensity and the intensity after passing through thickness  $l$ ;  $k$  is a constant. The absorption function  $r_\lambda$  and the scattering function  $s_\lambda$  are mutually dependent but may vary to different extents with the characteristics of the absorbing layer and with wave length. In such a layer the counterpart of the Bouguer-Lambert absorption law is

$$I_l = Ie^{-al} \quad (13-2)$$

where the attenuation coefficient  $a = k(r_\lambda, s_\lambda)$ .

near  $0.25\ \mu$ , after which it rises toward a new maximum. The general shape of the curves, particularly the maximum at  $0.28\ \mu$ , resembles that of most unconjugated proteins. It may be concluded that the protein component is principally responsible for the absorption.

The true attenuation in the epidermis is difficult to estimate because of the high degree of scattering. Different methods have been used for such measurements, those that are optically satisfactory yielding results in

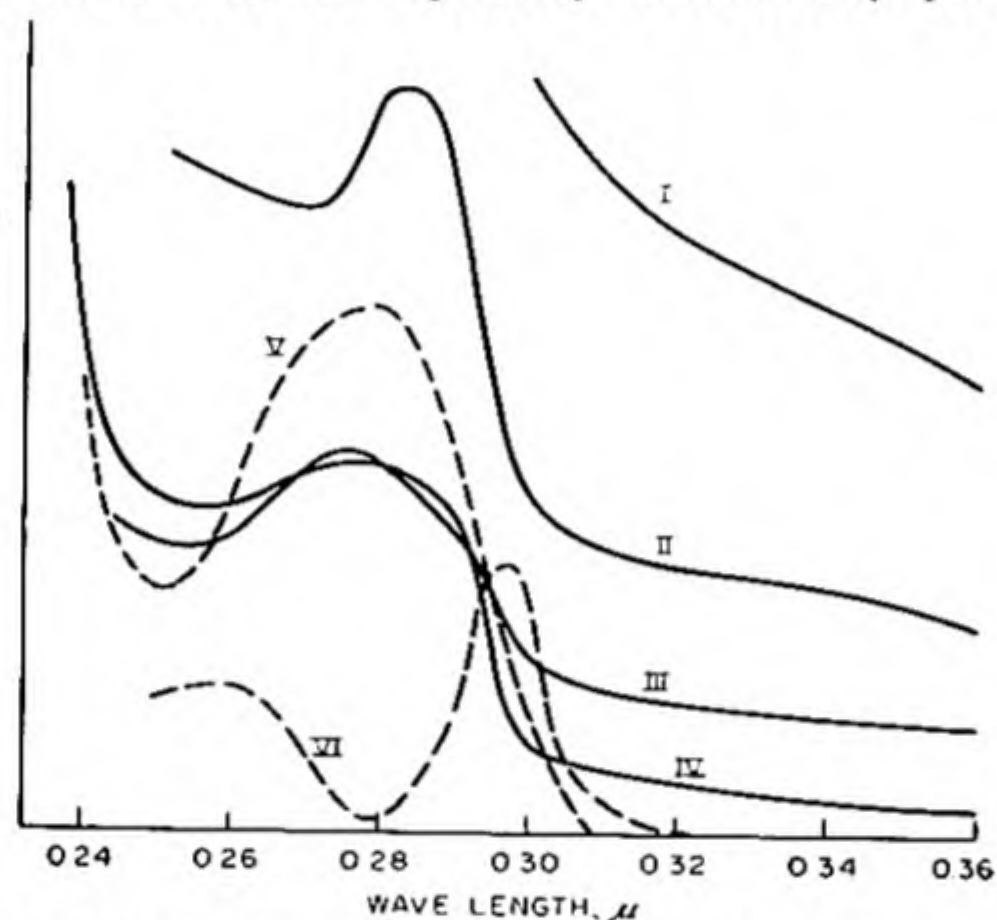


FIG. 13-2. Absorption by human epidermis. (After Blum, 1941a.)

Ordinate units are chosen for convenience to bring the data into comparison. The curves for epidermal attenuation, Nos. I, II, III, and IV, are plotted in terms of  $\log I_0/I$  in the same units, and are directly comparable. The data are from Lucas (1931) for a sample of human epidermis 0.08 mm thick. Curve I is measured in water, incident light parallel; this curve is dominated by scattering. Curve II, cleared in glycerin, incident light parallel. Curve III, cleared in acetic acid, incident light parallel. Curve IV, cleared in acetic acid, incident light scattered.

Curve V is a typical protein absorption spectrum plotted in terms of  $\log I_0/I$  but with a different unit. Curve VI is the erythema spectrum plotted as the reciprocal of the erythema threshold ( $1/Q_T$ ). (After Coblenz *et al.*, 1932.)

good general agreement (e.g., Lucas, 1931; Pearson and Gair, 1931; Kirby-Smith *et al.*, 1942; for additional references including other regions of the spectrum see Blum, 1945). Most measurements have been made on whole epidermis, including both the corneum and malpighian layer. It is possible by various means to strip off the whole epidermal layer (Baumberger *et al.*, 1942), but separation of the two layers of the epidermis has not been feasible. Pearson and Gair's measurements of the transmission of bits of corneum separated from sunburned skin indicate that the greater part of the incident erythema radiation is absorbed in this layer, and the character of the corneum and malpighian layer also suggest this (Kirby-Smith *et al.*, 1942). Therefore, probably only a relatively small fraction



reaches the malpighian layer itself, where the principal photochemical reaction of sunburn takes place. Rottier (1952) arrives at a similar picture from reflection measurements. An exact determination of the absorption in that layer when *in situ* would require that its thickness and that of the corneum, as well as the attenuation coefficients, be accurately known. The corneum is formed from dead cells which have been pushed up from the malpighian layer as new cells are formed there; the elements of the corneum which so effectively scatter the radiation represent the casts of these dead cells. There is no very exact line of demarcation between the two layers, the thicknesses of which vary from place to place on the body of a given person and from person to person. Various factors cause thickening of both layers, one of which is exposure to ultraviolet radiation. The effect of this on the erythema threshold will be discussed later.

Of particular interest with regard to the erythema spectrum is the correspondence of the maximum of absorption at  $0.28\ \mu$  with the sharp minimum in the erythema spectrum, clearly shown in Fig. 13-2. The strong absorption by the corneum in the region of  $0.28\ \mu$  probably accounts to a considerable extent for the minimum of effectiveness of these wave lengths in producing erythema, a point first made by Hausser in 1928. It is obvious that with a spectrally selective absorbing layer (the corneum) superficial to that in which the photochemical reaction occurs (the malpighian layer) the measured erythema spectrum does not reflect directly the nature of the absorbing substance which is concerned in that reaction. To reach the true action spectrum at the level of the malpighian layer, it would be necessary to make appropriate corrections for the attenuation of the radiation by the corneum, but this can be done only roughly. Some attempts at this will be discussed later on, but the inexactness of the measurements on which they must be based should not be lost sight of.

The interpretation of the erythema spectrum is further complicated by the findings of Rottier (1952, 1953) and Rottier and Mullink (1952), who present evidence that the corneum participates actively in the erythema response. They suggest that two photochemical reactions are concerned, one in the corneum and one in the malpighian layer. Dilator substance is contributed to the erythema response by both reactions, but, since there appear to be two different light absorbers involved, the dilator substances presumably differ also. These investigators suggest that the light absorber in the corneum may be a sterol. This active participation of the corneum does not, of course, prevent it from acting also as a spectral filter for the radiation reaching the malpighian layer, so that what has been said here about the effect of absorption by the corneum is still pertinent. It simply adds one more complicating factor that must be taken into account.

*Factors Affecting the Erythema Threshold.* There are various instances in which it would be convenient to have a standard erythema threshold that would apply to the "average" person. This would be particularly useful in comparing the effects of different polychromatic sources, e.g.,

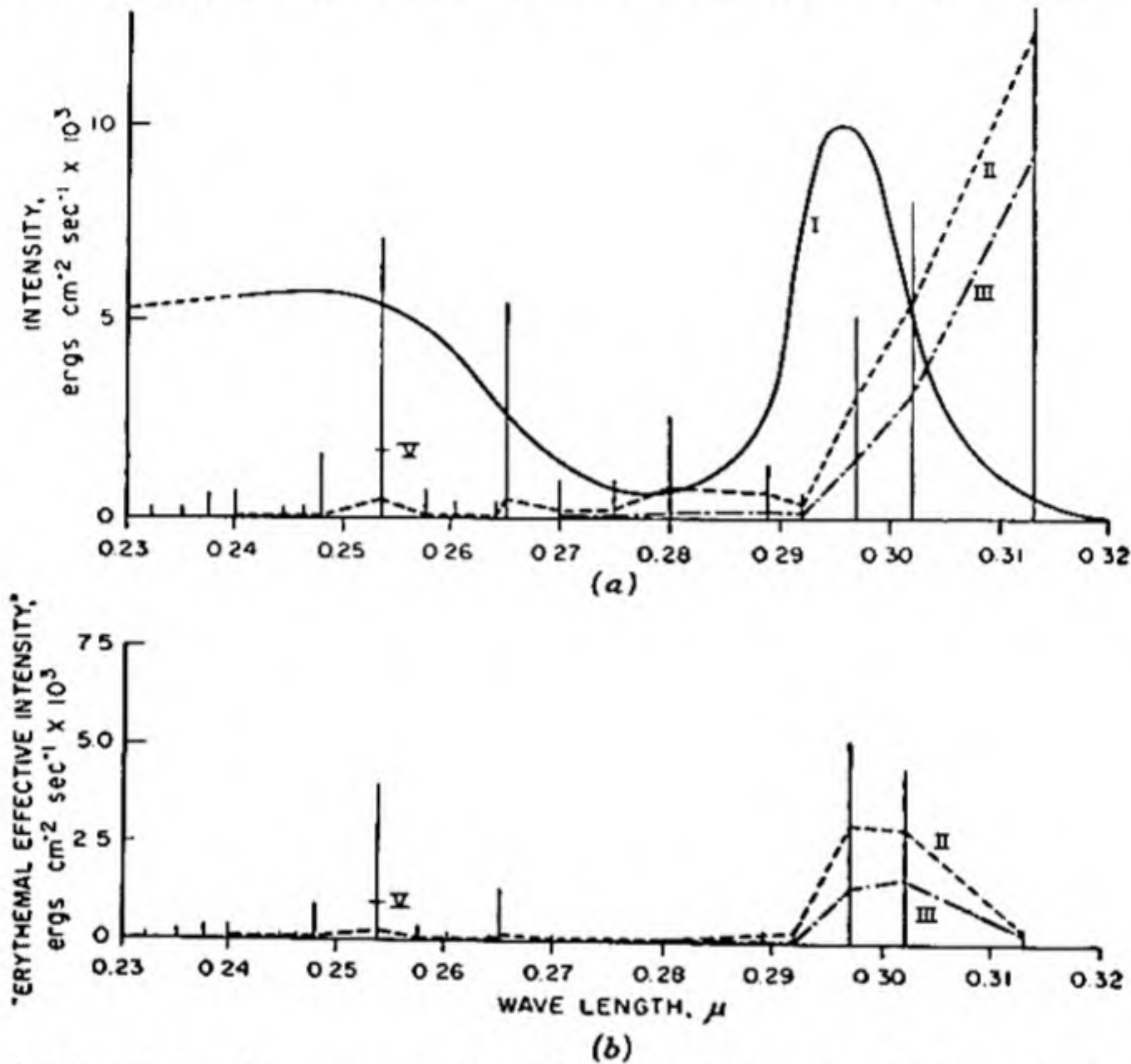


FIG. 13-3. Spectrum of a mercury arc (diagrammatic); (a) absolute intensities; (b) "erythema effective intensities," i.e., the absolute intensities corrected for the erythema spectrum shown at I in (a). The height of each vertical line represents the intensity of radiant flux of this wave length (intermediate-pressure mercury arc). The points where curve II cuts the vertical lines represent the intensities when a Corex D filter was present. The points where curve III cuts the vertical lines represent the intensities when a pyrex filter was present. Point V on the line at wave length 0.2537  $\mu$  represents the intensity of a low-pressure mercury arc, which may be regarded as emitting only this wave length. (After Blum and Terus, 1946a.)

natural sunlight and radiation from the mercury arc. On first consideration, it might be expected that a reasonable approximation could be made in terms of the erythema spectrum, and there have been various attempts to set such a standard.

Let us represent the total amount of incident radiation within the erythema spectrum by the symbol  $Q$  and the threshold quantity of such radiation by  $Q_r$ . Correspondingly, for monochromatic radiation we may use the symbols  $Q_\lambda$  and  $Q_{\lambda r}$ . In Fig. 13-3a is diagrammed the spectrum of a mercury arc in the erythema region. The spectrum is composed of dis-



crete monochromatic lines. For any given line of wave length  $\lambda$ ,

$$Q_{\lambda} = It \tag{13-3}$$

where  $I$  is the incident intensity of the line and  $t$  the duration of the exposure.<sup>15</sup> The total incident energy from the mercury arc within the limits of the erythema spectrum may be obtained by summing the amounts of energy delivered by the individual lines and multiplying by  $t$ :

$$Q = t(I_1 + I_2 + \dots + I_i) \tag{13-4}$$

where  $I_1, I_2, \dots, I_i$  are the intensities of the various lines.

In Fig. 13-3*b* the “erythema effective intensity” of the lines is indicated. This is obtained by multiplying the intensity of each line by the

TABLE 13-1. ERYTHEMAL THRESHOLD IN TERMS OF “ERYTHEMAL EFFECTIVE ENERGY”<sup>a</sup>  
(Blum and Terus, 1946a.)

Subject	Effective energy, $\times 10^3$ ergs/cm <sup>2</sup>			
	Intermediate-pressure mercury arc			Low-pressure mercury arc, 0.2537 $\mu$
	No filter	Corex D filter	Pyrex filter	
1	56	262	...	33
2	45	218	141	30
3	45	248	212	28
4	147	525	324	66
5	64	240	...	29
6	56	335	...	45
7	118	320	554	62
	76 (avg.)	307	308	41

<sup>a</sup> See Fig. 13-3*a* and *b*.

erythema sensitivity  $S$  for that wave length relative to the maximum in the erythema spectrum at 0.2967  $\mu$  (see Fig. 13-3*a*; the relative sensitivity is, of course, measured in terms of the reciprocal of the threshold). We may then express the “erythema effective energy”  $Q'$  as

$$Q' = t(I_1S_1 + I_2S_2 + \dots + I_iS_i). \tag{13-5}$$

This is a measure which has been variously employed. Let us accept it as an approximation which can be put to test. In Fig. 13-3 are shown spectra of the mercury arc when restricted with spectral filters, and also the intensity of a low-pressure mercury arc which delivers practically all its radiation in the 0.2537- $\mu$  line. In an experiment carried out by Blum

<sup>15</sup> It is understood that the values of  $Q$  and  $I$  are referred to unit area of the skin upon which they impinge, but for brevity of discussion this will not be specifically stated in each case.

and Terus (1946a), thresholds were measured on a number of subjects under these different conditions and calculated in terms of Eq. (13-5). The results summarized in Table 13-1 show that the approximation is not a very close one. The threshold values are of the same order of magnitude, but vary systematically according to the source, being higher when only the longer wave lengths are present.

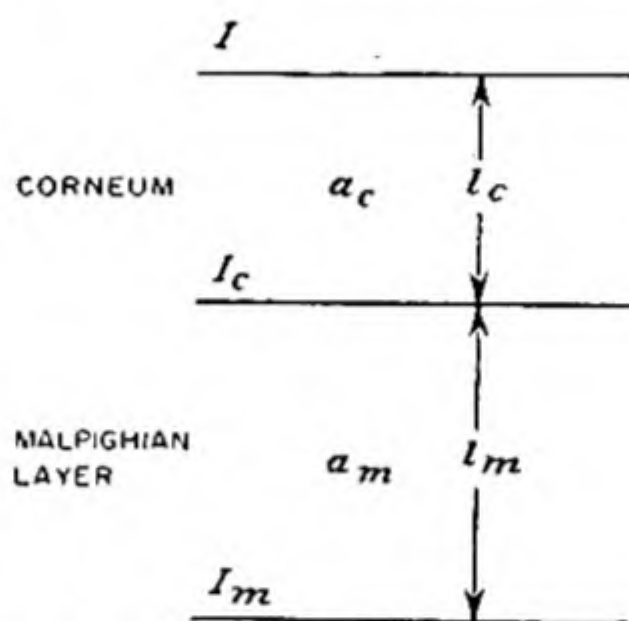


FIG. 13-4. Diagrammatic representation of the epidermis.  $I$ ,  $I_c$ , and  $I_m$  are intensities at the levels indicated;  $l_c$  and  $l_m$  are the thicknesses of the layers indicated;  $a_c$  and  $a_m$  are the attenuation coefficients of the layers indicated. (After Blum and Terus, 1946b.)

A simplified model may help us in inquiring into the causes of such deviation. Let us indicate by  $D$  the quantity of dilator substance elaborated in the malpighian layer and resulting in grossly observed erythema. Then for a given wave length  $\lambda$ , we may write

$$D = \frac{At}{hc/\lambda} \gamma \quad (13-6)$$

where  $A$  represents the amount of radiant energy of wave length  $\lambda$  absorbed per unit of time by the light absorber for the photochemical reaction leading to the formation of dilator substance, and  $t$  is the duration of the exposure. The quantity  $At$ , like  $Q_\lambda$ , is measured in units of energy, whereas the amount of  $D$  formed must be a function of the number of quanta absorbed; this number is obtained by dividing by the energy of the quantum,  $hc/\lambda$ , where  $h$  is Planck's constant and  $c$  is the velocity of light. The coefficient  $\gamma$  is the ratio of molecules of dilator substance formed to the number of quanta absorbed, corresponding to the efficiency or quantum yield of any photochemical reaction; it may or may not vary with wave length.

Let us assume for convenience that the corneum and the malpighian are sharply separated homogeneous layers which may be represented as in Fig. 13-4. The intensity at the bottom of the corneum when a beam of



intensity  $I$  impinges upon the skin surface is  $I_c$ , and  $I_m$  is the intensity at the bottom of the malpighian layer. The difference between  $I_c$  and  $I_m$  must represent the amount of radiation absorbed plus the amount scattered per unit time in the malpighian layer, and of this  $A$  is some definite fraction. We may therefore write

$$A = (I_c - I_m)\beta \quad (13-7)$$

where  $\beta$  is a proportionality factor which may be different for different wave lengths. The light absorber is assumed to be present in such large amount that its concentration is virtually unchanged during exposure.

If  $l_c$  is the thickness of the corneum and  $a_c$  the attenuation coefficient (see footnote 14) of that layer, we may write

$$I_c = Ie^{-a_cl_c} \quad (13-8)$$

Similarly,

$$I_m = Ie^{-a_m l_m} \quad (13-9)$$

where  $a_m$  is the attenuation coefficient and  $l_m$  the thickness of the malpighian layer. Combining Eqs. (13-8) and (13-9),

$$I_m = Ie^{-a_cl_c}e^{-a_m l_m} \quad (13-10)$$

and substituting from Eqs. (13-8) and (13-10) in Eq. (13-7), we obtain

$$A = I[e^{-a_cl_c}(1 - e^{-a_m l_m})\beta]. \quad (13-11)$$

Combining Eq. (13-11) with Eqs. (13-3) and (13-6),

$$Q_\lambda = \frac{Dhc}{[e^{-a_cl_c}(1 - e^{-a_m l_m})\beta]\gamma\lambda} \quad (13-12)$$

Then for polychromatic radiation,

$$Q = hc \left\{ \frac{D_1}{[e^{-a_cl_c}(1 - e^{-a_m l_m})\beta]_1\gamma_1\lambda_1} + \frac{D_2}{[e^{-a_cl_c}(1 - e^{-a_m l_m})\beta]_2\gamma_2\lambda_2} + \dots + \frac{D_i}{[e^{-a_cl_c}(1 - e^{-a_m l_m})\beta]_i\gamma_i\lambda_i} \right\}. \quad (13-13)$$

We note in this expression that the intensity terms for the various lines have dropped out.<sup>16</sup> Hence there is nothing to indicate why the discrepancies in Table 13-1 should appear when the relative intensities of the lines are changed by interposing filters, as indicated in Fig. 13-3. Differences in absorption in the corneum and malpighian layer, represented by the terms within the brackets [see Eq. (13-11)] would be the same for a given individual and hence would not account for such discrepancies. Variation in the coefficient  $\gamma$  with wave length would alter the shape of the

<sup>16</sup> It will be seen that reciprocity, which is implicit in these equations, is obeyed for monochromatic radiation.

"standard" erythema spectrum in the first place, but would not account for the discrepancies when different spectral sources were used. The source of these discrepancies must be sought elsewhere.

In all this discussion it is tacitly understood that the threshold is determined by the elaboration of a given amount,  $D_r$ , of the dilator substance, which presumably brings about a minimal detectable dilation of the minute vessels. If this were true at the threshold condition,

$$D_r = D_1 + D_2 + \cdots + D_i. \quad (13-14)$$

This assumption seems obvious enough since it would not be anticipated, on first consideration, that the amount of response of the vessels to a given



FIG. 13-5. Diagram showing the result of exposure to a series of doses of "long-wave-length" (filtered through pyrex) mercury arc radiation as follows:

- 1:  $2.4 \text{ ergs cm}^{-2} \times 10^5$  of "erythema effective energy"
- 2:  $2.7 \text{ ergs cm}^{-2} \times 10^5$  of "erythema effective energy"
- 3:  $3.4 \text{ ergs cm}^{-2} \times 10^5$  of "erythema effective energy"
- 4:  $5.3 \text{ ergs cm}^{-2} \times 10^5$  of "erythema effective energy"
- 5:  $8.0 \text{ ergs cm}^{-2} \times 10^5$  of "erythema effective energy"
- 6:  $12.0 \text{ ergs cm}^{-2} \times 10^5$  of "erythema effective energy"
- 7:  $18.1 \text{ ergs cm}^{-2} \times 10^5$  of "erythema effective energy"
- 8:  $26.6 \text{ ergs cm}^{-2} \times 10^5$  of "erythema effective energy"
- 9:  $40.0 \text{ ergs cm}^{-2} \times 10^5$  of "erythema effective energy"

The amount of shading indicates the degree of erythema 2 hr after the exposure. Note that the erythema is less pronounced for the highest doses than for some of the intermediate doses. (After Blum and Terus, 1946a.)

amount of dilator substance varies with the wave length. However, it is apparently necessary to assume the latter in order to account for the discrepancies in Table 13-1.

Blum and Terus (1946a) offered an explanation of this discrepancy based on their finding that large doses of the longer wave lengths of the erythema spectrum could cause inhibition of the erythema brought about by these or shorter wave lengths. Figure 13-5 indicates diagrammatically the various degrees of erythema 2 hr after exposure to a graded series of doses of ultraviolet radiation including both the longer and shorter wave lengths of the erythema spectrum. The degree of erythema increases with increasing dose up to a certain point and then falls off as higher doses are given. This apparent optimum was not observed when only shorter wave lengths of the erythema spectrum were used (i.e.,  $0.2537 \mu$ ). The



subsequent blistering and desquamation that followed the high doses of the longer wave lengths likewise showed no such optimum. It therefore seems that only the erythema is inhibited, not the underlying photochemical reaction as reflected in other aspects of sunburn. This point of view was supported by results of further experiments. Doses of short-wave-length erythematous radiation ( $0.2537 \mu$ ) sufficient to elicit erythema were first given (A,B,C). Then, before the erythema had had time to develop, doses of the longer wave lengths of the erythematous spectrum ( $\sim 0.29\text{--}0.32 \mu$ ) were applied across the same areas (D,E,F). The

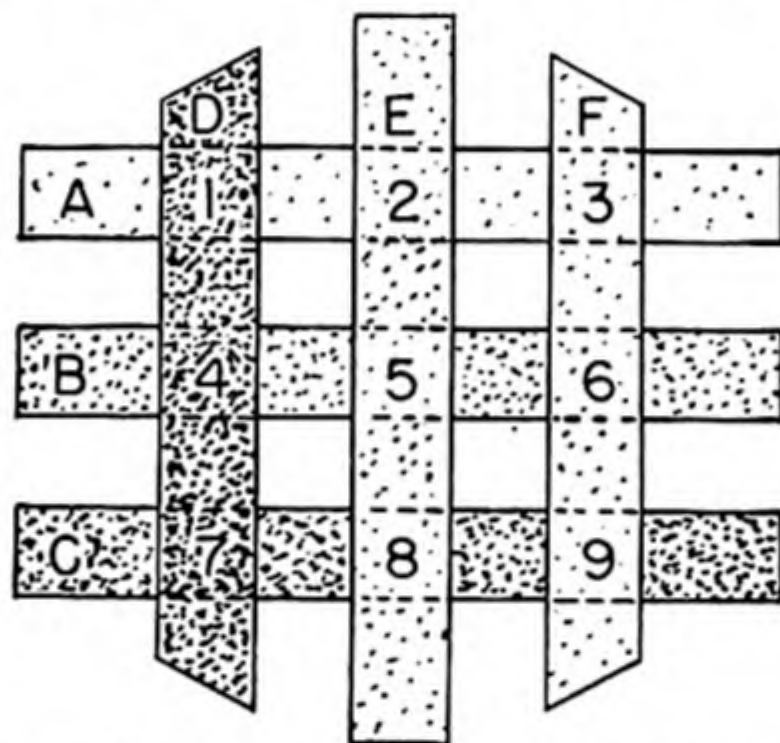


FIG. 13-6. Result of exposure to the longer wave lengths of mercury arc (filtered through pyrex) (D,E,F), after exposure to wave length  $0.2537 \mu$  (A,B,C).

A:  $1.1 \text{ ergs cm}^{-2} \times 10^5$  of "erythematous effective energy"

B:  $3.9 \text{ ergs cm}^{-2} \times 10^5$  of "erythematous effective energy"

C:  $8.3 \text{ ergs cm}^{-2} \times 10^5$  of "erythematous effective energy"

D:  $15.4 \text{ ergs cm}^{-2} \times 10^5$  of "erythematous effective energy"

E:  $35.2 \text{ ergs cm}^{-2} \times 10^5$  of "erythematous effective energy"

F:  $52.2 \text{ ergs cm}^{-2} \times 10^5$  of "erythematous effective energy"

Doses D, E, and F were applied approximately 15 min after A, B, and C. The diagram represents the various degrees of erythema  $2\frac{1}{2}$  hr after the last exposure. Note inhibition of erythema in areas 5, 6, 8, and 9. (After Blum and Terus, 1946a.)

latter inhibited the erythema ordinarily resulting from the former, as indicated in Fig. 13-6. The whole of the areas that received the heavy doses of the longer-wave-length radiation subsequently underwent blistering and desquamation.

The inhibition of erythema without inhibition of sunburn as a whole, was interpreted as resulting from a direct effect of the longer wave lengths of the erythematous spectrum on the minute vessels of the skin, limiting their response to the dilator substance formed in the epidermis. The transmission spectra for human epidermis presented in Fig. 13-7 show that the longer wave lengths of the erythematous spectrum do penetrate below the epidermis to a certain extent and could act directly on the minute vessels. The inhibition of erythema would seem comparable to the inhibition of

flushing after severe superficial burn from heat, when the skin may be blanched in the area of the burn. Although the inhibition of erythema is experimentally demonstrable only with severe doses of these longer wave lengths of the erythema spectrum, it seems reasonable to assume that they exert some inhibitory effect even at much lower doses and hence may influence the erythema threshold when determined with polychromatic radiation. Partial inhibition of dilation of the minute vessels by the longer wave lengths would raise the observable erythema threshold to

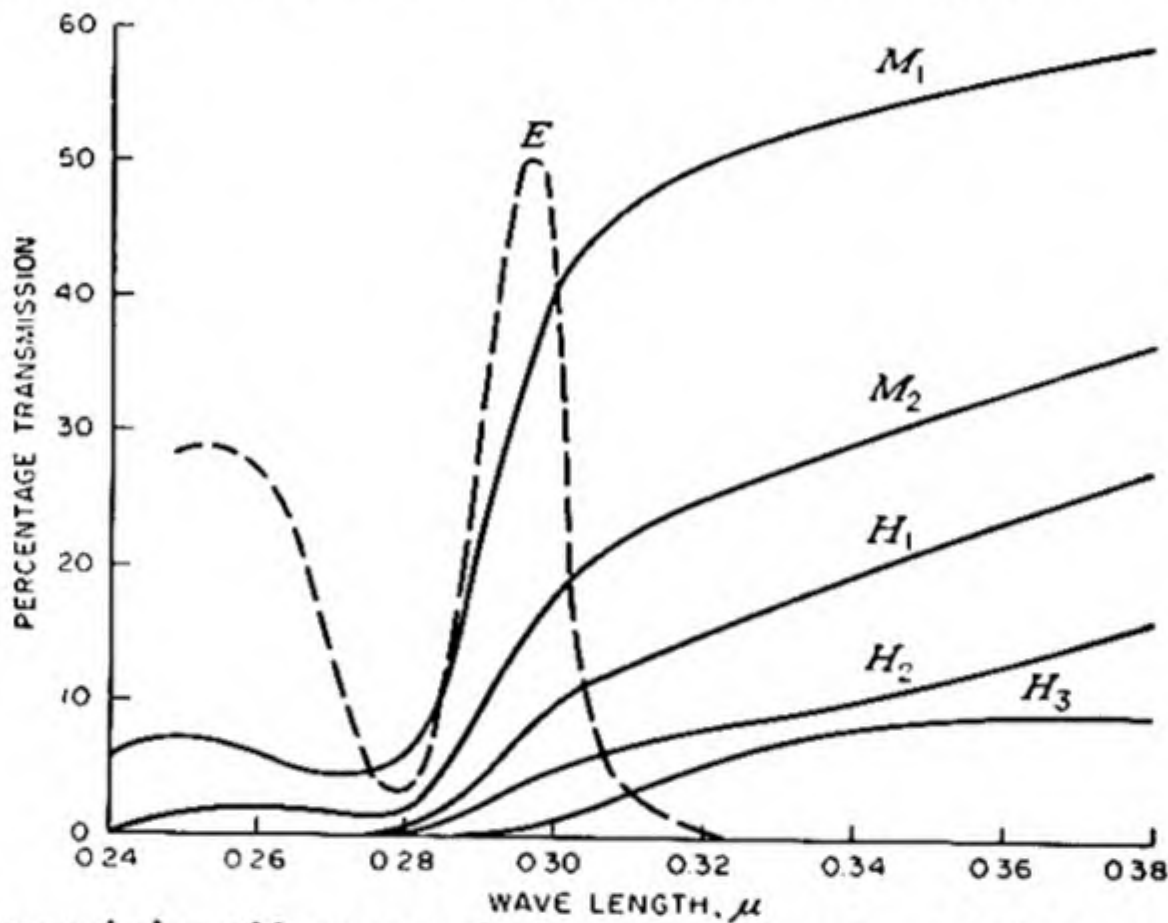


FIG. 13-7. Transmission of human and of mouse epidermis.  $M_1$  is from ear of normal young mouse;  $M_2$  from ear of mouse subjected to repeated exposures to ultraviolet radiation.  $H_1$  from untanned volar surface of human forearm;  $H_2$  from slightly tanned volar surface of the same forearm;  $H_3$  from heavily tanned dorsal surface of forearm. All measurements are for transmitted radiation collected at  $45^\circ$ , epidermis not cleared. The transmitted radiation collected at  $180^\circ$  would be somewhat greater. (After Kirby-Smith et al., 1942.)

these wave lengths. This would in effect negate Eq. (13-14) by making the response to the dilator substance effectively wave-length dependent.

Another explanation may now be based on the finding by Rottier (1952) and Rottier and Mullink (1952) that dilator substance is formed in the corneum. Our simple model does not take this into account, but could be extended to do so by making additional assumptions. If the dilator substance formed in the corneum were different from that formed in the malpighian layer and its action on the vessels differed quantitatively, or if the rate of formation and diffusion to the vessels were quite different in the two cases, the relation in Eq. (13-14) need not hold. This is another possible explanation of the discrepancy that has been bothering us; perhaps both explanations are pertinent to the complex response studied in sunburn.



In the model described, reciprocity ( $I \times t = \text{a constant}$ ) has been assumed; it is specifically stated in Eq. (13-3) and is implicit in Eq. (13-6). Experimentally, reciprocity does hold over a wide range of doses when monochromatic radiation is used to determine the threshold, as has been shown by Hausser and Vahle (1922), Coblentz *et al.* (1932), and Blum and Terus (1946b). On the other hand, when polychromatic radiation is used there may be a wide deviation from reciprocity, as shown by Schall and Alius (1926, 1928a) and by Blum and Terus (1946b). This would seem to reflect complex time relationships in the mechanism of vascular dilation.

The existence of more than one factor active in determining the erythema spectrum also explains certain of Hausser's findings (1928) that had always remained puzzling. When he studied the erythema effectiveness in terms of the intensity of the erythema, Hausser found that for shorter wave lengths a proportional increase in dosage produced a relatively greater increase in intensity of the erythema than did a comparable increment of the longer wave lengths. This is difficult to understand in terms of a simple model, but is not surprising in a complex system involving processes with different wave-length dependence.

Various factors have been reported to affect the erythema threshold, but when these reports are based on minor differences they should be accepted with caution. Threshold measurements are of limited accuracy at best, and the great individual variation makes statistical treatment uncertain in view of the obvious complexity of the erythema mechanism. Heat is one of the factors that might be expected to affect the erythema response, but, although numerous studies have been made, the results are somewhat conflicting. Schall and Alius (1928a) and Clark (1936) both found that the temperature during exposure to ultraviolet radiation had very little effect on the threshold. Clark found, on the other hand, that the rate of appearance of the erythema increased with temperature. This is explained if we assume that the primary photochemical reaction is, as might be expected, virtually independent of temperature, but that subsequent parts of the erythema process are temperature dependent. Failure to separate these two phases of the response may account for some of the apparent disagreement in results obtained by other investigators. Recently Helmke (1948-49) reported that infrared radiation lowers the erythema threshold and shortens the time to appearance of erythema in a majority of individuals.

### SUNTAN

*Pigment Migration and Formation.* Observed grossly, the erythema of sunburn appears to fade almost imperceptibly into suntan; i.e., there is a gradual color change from red to brown. But this does not represent an immediate relation between these two aspects of sunburn. Whereas the

former results from the increased blood flow in the minute vessels of the dermis, the latter represents the change in position and increase in quantity of melanin pigment in the epidermis. That the color of suntan is principally due to melanin is indicated by studies of the spectral distribution of the radiation reflected from tanned and untanned skin (Edwards and Duntley, 1939a, b). The difference in position of the erythema and tan may be readily demonstrated by pressing a sheet of glass against the skin. This squeezes the blood out of the minute vessels, causing the red color to disappear, but the tan remains visible since the melanin pigment in the epidermis is not affected. In this way the time of disappearance of erythema and the appearance of tan may be judged.

In untanned skin the melanin pigment is located chiefly in the basal cell layer (e.g., Masson, 1948). According to histological studies, about the time that tan first becomes grossly observable, this pigment begins to migrate toward the surface, where it should have a greater effect on the spectrum of the reflected light from the skin's surface. The pigment migration begins about 24 hr after the initial exposure to ultraviolet radiation. It was first described by Keller (1924a) (*Pigmentverschiebung*). It was also noted by Peck (1930) in the case of pigmentation brought about by ionizing radiation from thorium (pseudohyperpigmentation) and is suggested in earlier studies by Lutz (1917-18). The mechanism of this migration of melanin does not seem to be clearly explained. Recent spectrophotometric studies by Jansen (1953) suggest that there is early pigment formation preceding the migration.

Later there is increase of melanin in the region of the basal cell layer, the amount depending on the severity of the dose and whether repeated (Lutz, 1917-18; Keller, 1924a, b; Hamperl *et al.*, 1939a). While it has been maintained that all the basal cells can form melanin, the point of view that only certain dendritic cells accomplish this gains favor (e.g., Masson, 1948). The pigment may be found in the prickles cells of the malpighian layer and in the corneum of skin that has been subjected to repeated doses of ultraviolet radiation.<sup>17</sup> Once formed, the pigment persists in the epidermis for months and may still be grossly observable after several years. In later discussion the total of changes involving the melanin pigment will be referred to as melanization, pigment migration and pigment formation being distinguished as parts of the complex.

*Pigment Darkening.* Formerly it was thought that tanning is brought about by ultraviolet wave lengths somewhat longer than those which elicit erythema. But in 1939 Henschke and Schultze published a series of studies which showed that, while such wave lengths do cause darkening of the skin, the mechanism is quite different from that of the primary melani-

<sup>17</sup> What has been said applies to the skin of the white races. In Negro skin the pigment is more uniformly distributed throughout the epidermis, including the corneum, but comparable histological studies are lacking.



zation of the epidermis. About a year earlier I. Hausser (1938) had reported that a dark brown coloration of normal skin is brought about by wave lengths longer than those which produce erythema. Henschke and Schultze studied this phenomenon extensively, coming to the conclusion that it represents the darkening of preformed melanin; it will be referred to here as "pigment darkening." This differs in many respects from the primary melanization that is brought about by the erythema spectrum, i.e., by wave lengths shorter than about  $0.32\ \mu$ . The action spectrum for pigment darkening extends from about  $0.3\ \mu$  to about  $0.42\ \mu$  with a broad maximum near  $0.34\ \mu$ , as is represented in Fig. 13-8. In contradistinction to erythema and pigment formation, pigment darkening is readily brought about by sunlight passing through window glass, which by removing the wave lengths shorter than  $0.32\ \mu$ , prevents both erythema and primary

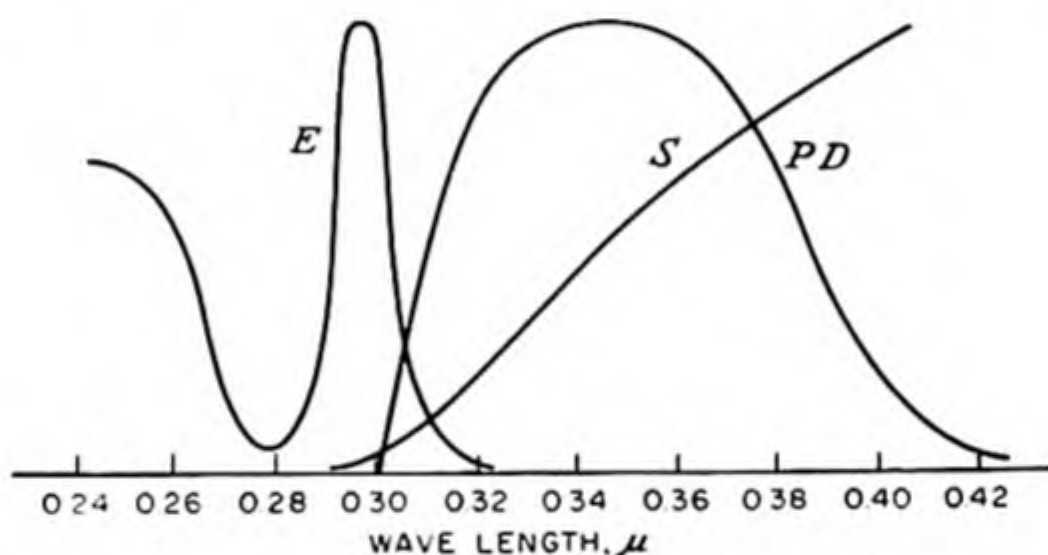


FIG. 13-8. *PD*, action spectrum for pigment darkening. *E*, erythema spectrum; *S*, sunlight. All ordinates are arbitrarily chosen and do not indicate relative magnitudes. (After Henschke and Schultze, 1939a.)

melanization of the epidermis.<sup>18</sup> Pigment darkening may appear within the first few minutes of exposure to sunlight and usually reaches its maximum within an hour, whereas pigment migration does not begin for at least 24 hr, and new pigment formation only after a few days. Several-hundredfold greater dosages of radiant energy are required to bring about pigment darkening than are necessary for erythema and primary melanization. Pigment darkening is most pronounced in skin that has been previously sunburned and still retains traces of suntan, whereas melanization is greatest in skin not previously sunburned. Pigment darkening does not occur when oxygen has been removed by blanching the skin by pressing a quartz plate against it, whereas erythema and melanization are not affected by this treatment. Histological examination shows that these longer wave lengths do not cause pigment migration or the forma-

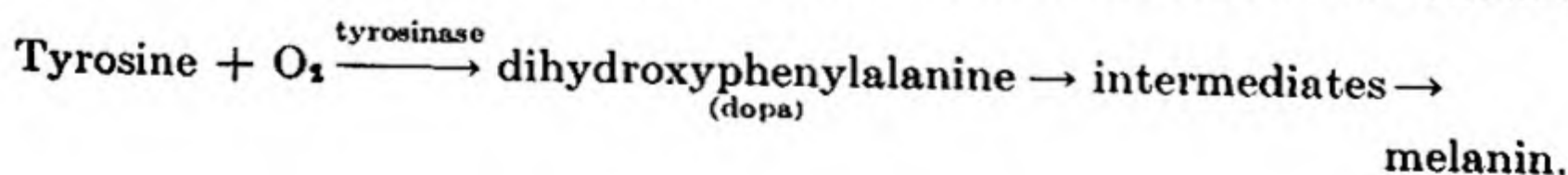
<sup>18</sup> Common window glass, which is a fairly standard product, cuts out almost all the wave lengths shorter than  $0.32\ \mu$ . A person therefore does not ordinarily become sunburned by sunlight passing through a window pane, although it is possible to elicit an erythema in this way with intense midsummer sunlight. There have been window glasses on the market in recent years which transmit somewhat more of the erythema radiation.

tion of new melanin (Hamperl *et al.*, 1939a). The failure to differentiate the processes of melanization and pigment darkening has led to confusion and to the belief that primary melanization is brought about by wave lengths longer than those of the erythema spectrum.

Miescher and Minder (1939) confirmed and extended the findings of Henschke and Schultze, offering an explanation in terms of the Meirowsky effect. Meirowsky in 1909 and later Lignac (1923) had found that darkening of the pigment of cadaver skin is brought about by ultraviolet radiation or by heat, and that such darkening does not occur in the absence of oxygen. Miescher and Minder pointed out that the pigment darkening in living skin parallels these findings, and presented further evidence of the identity of these effects. They postulated that the darkening represents the oxidation of melanin pigment already present in the skin in a reduced leuko form. The exact nature of this leuko form was not specified, but Figge (1939) has since shown that melanin may be reversibly bleached in an *in vitro* oxidation-reduction system. The writer has suggested (1945) that the melanoid pigment described in human skin by Edwards and Duntley (1939a, b), as a result of studies of spectral reflection, is a leuko form of melanin, or a mixture of the oxidized and reduced forms. Sharlit (1945) has vehemently attacked the findings and conclusions of Miescher and Minder. Unfortunately, he does not give any description of the ultraviolet radiation he used nor does he indicate that he recognized either the difference between pigment formation and pigment darkening or the spectral difference of the radiation bringing about the two effects. It is to be hoped that this discrepancy in results will be explained by further experimental study.

*Hormones and Tanning.* That hormones may influence the tanning of human skin was first shown by Hamilton and Hubert (1938), who found in eunuchoids that previously sunburned areas darkened in color when androsterone was injected. Although other factors are concerned, including circulatory changes, melanin pigment is involved in this phenomenon (see Hamilton, 1948).

*Biochemical Aspects.* *In vitro* reactions leading to the formation of melanin have been applied with considerable success to melanin formation in certain species, but until recently there have been objections to interpreting suntanning in terms of such reactions. There are still uncertainties to be explained, but the resolution of some of the difficulties seems to be in sight. The general subject has been recently reviewed by Lerner and Fitzpatrick (1950), and there will be no attempt to do so here. Let us assume, tentatively, that the melanin formation associated with suntan follows the same chemical steps as the *in vitro* reaction, which may be schematized in abbreviated form suitable to this discussion as follows:





The amino acid tyrosine is oxidized to dihydroxyphenylalanine, commonly known as "dopa," by the action of the enzyme tyrosinase, a copper protein complex. Molecular oxygen takes part in the first step *in vitro* and again in later steps. There seems no objection to applying such a scheme to melanin formation in suntanning, but difficulty arises when further attempt is made to represent the melanin formation, let alone the whole process of suntanning, in terms of a photochemical forwarding of this *in vitro* reaction scheme.

In 1933 Frankenburger found that exposure of solutions of tyrosine to ultraviolet radiation resulted in the formation of a brown pigment, presumably melanin, but very large doses of radiation were required. Later Arnow (1937) found that ultraviolet radiation could bring about oxidation of tyrosine to dopa, but again the amount of radiation required was much greater than would be needed for the *in vivo* production of suntan. Rothman showed in 1942 that this reaction may be accelerated by the presence of small amounts of ferrous salts and that when these are present the quantities of energy required are nearer to those effective in suntanning. The possibility must be considered that ultraviolet radiation acts *in vivo* to bring about the oxidation of tyrosine to dopa in a manner parallel to the *in vitro* reactions. After this, the succeeding steps may presumably take place without the intervention of ultraviolet. There seems one objection to all these schemes in that the *in vitro* production of melanin, and specifically the initial step, are aerobic, whereas *in vivo* the reduction of the molecular oxygen supplied by occlusion of the circulation to the skin during the exposure to ultraviolet radiation has no effect on the subsequent melanization (Blum *et al.*, 1935; Henschke and Schultze, 1939a; Blum, 1941b). It may be objected that by such methods the partial pressure of oxygen in the skin is not reduced sufficiently to inhibit the reaction. Sharlit (1945), as a result of his studies on cadaver skin, suggested that oxygen is obtainable from the cytochrome system. It can only be pointed out that the same method of removing oxygen inhibits pigment darkening and photosensitized oxidations. This point need not be emphasized, however, since there are other difficulties to consider.

Rothman *et al.* (1946) have proposed a scheme which is not based directly on the *in vitro* reactions and so may avoid the above objection. They found for human skin, as had Ginsberg (1944) for the guinea pig, that there is present a factor which inhibits melanin formation *in vitro*. This they identified tentatively as water-extractable sulfhydryl compounds. They suggest that ultraviolet radiation and other injurious agents cause destruction of these compounds with the result that the formation of melanin can proceed. Fitzpatrick *et al.* (1949, 1950) include Rothman's scheme to account for the melanin formation of suntan. They also include the oxidation of tyrosine to dopa by direct photochemical effect, and add another factor, the acceleration of this reaction

by the formation of dopa itself, which they have found to be catalytic in trace amounts. The photochemical formation of dopa meets, of course, the same difficulty mentioned, since, presumably, oxygen must be present during the exposure to ultraviolet radiation. It may be pointed out that this is not an objection to the *in vitro* reaction scheme as a whole since oxygen is present during the time when the melanin is actually formed, but only to the acceptance of the photooxidative step as the initial one.

But it seems that none of these schemes, except possibly that of Rothman, takes cognizance of what happens in suntanning. None accounts adequately for the delay of several days between the exposure to ultraviolet radiation and the first observable formation of new melanin. Whatever the ultraviolet radiation does, it must be thought of as setting off a chain of events that leads to melanin formation at a later time. The removal of inhibiting substances as suggested by Rothman might act in this way, but it is difficult to fit the other schemes into this picture, although the formation of small amounts of dopa, which then catalyze the formation of larger amounts, has attractive aspects. It may also be pointed out that the initial part of the melanization process—migration of pigment already formed—is not accounted for in any way by these schemes.

The objection may be levied against a good many of the *in vitro* studies that massive doses have been used which bear no relation to those which produce *in vivo* effects. There is more and more evidence to indicate that profound changes may be brought about in living cells by very small quantities of ultraviolet radiation. Thus, while ultraviolet may bring about in living skin the formation of a small amount of melanin according to reactions such as have been demonstrated *in vitro*, the skin might be pretty thoroughly cooked before the quantity reaches the proportions of suntan. On the other hand, a very small amount of ultraviolet radiation might, by eliciting changes in the cell, lead to the ultimate production of a good deal of melanin.

In this chapter the point of view is adopted that the primary effect of the ultraviolet radiation is injury to cells of the malpighian layer and that melanization, like other aspects of the sunburn mechanism as a whole, is secondary to this injury. Melanization is a common response of the epidermis to injury of any kind, which may represent an over-all reaction similar to or identical with that by which melanin is formed *in vitro*. But the initiating of such a reaction need not be specifically related to the agent that brings about the injury. In this regard the experiments of Peck (1930) may be cited, which show that melanization brought about by radiation from thorium closely parallels melanization from ultraviolet radiation, there being, in both cases, first pigment migration and then the elaboration of more pigment. There is little reason for believing that the ionizing radiation from thorium would bring about the same kind of



photochemical reaction that is postulated by the biochemical scheme just discussed, but both ionizing radiation and ultraviolet injure the epidermal cells. Moreover, melanization of human epidermis follows injury by such diverse agents as heat, rubbing, photodynamic action (photosensitized oxidation) and wounds. Unfortunately, there is no very good explanation for melanization of the epidermis caused by any of these agents, but, until such matters are more clearly understood, the idea of direct formation of melanin by photochemical reaction, attractive though it may be, should not be accepted without reservation.

### PHOTOSENSITIZATION

Sensitization of the skin to light by exogenous agents cannot be discussed here at length. It may be pointed out, however, that in human skin there are at least two different types of such action. As examples, sulfanilamide sensitization and the photodynamic action of eosin (the cause of photosensitization by lipstick) may be chosen. Sulfanilamide does not act as a true photosensitizer—i.e., it does not participate as the light absorber—but seems only to increase the injury to the skin brought about by ultraviolet radiation. Eosin, on the other hand, acts as a photosensitizer, absorbing the light and bringing about photochemical oxidation of skin constituents. Photosensitization by eosin is inhibited by occlusion of oxygen; photosensitization by sulfanilamide is not (Blum, 1941b). Photosensitization of human skin is discussed elsewhere by the author (1941a), and other aspects of photooxidation will be treated by Norman Clare in volume III of this series.

### PROTECTION AGAINST SUNBURN

*Natural Protection.* Following even a mild degree of sunburn the erythema threshold rises and may remain above its previous level for about two months. The decrease in sensitivity to ultraviolet radiation takes place at approximately the same time that suntan is developing. This observation and the relative insensitivity of Negro skin to sunlight led long ago to the idea that the melanin pigment serves to protect the skin against sunburn by absorbing the sun's rays (e.g., Davy, 1828; Wedding, 1887; Bowles, 1889). The experiment of painting the skin with an opaque material, e.g., India ink, and so obtaining protection from sunburn, has been repeatedly interpreted as supporting the idea of protection by the melanin pigment (e.g., Davy, 1828; Finsen, 1900) which persists as one of the most popular misconceptions regarding sunburn.

The idea apparently remained unchallenged until about 1920, after which objections were raised by a number of workers. Probably the first of these was With (1920) who found that areas of vitiliginous skin, which do not develop melanin pigment, can be rendered less sensitive to ultra-

violet radiation by repeated doses of this agent, an observation also made a few years later by Meyer (1924). Keller (1924b) noted that the first darkening of the skin results from migration of the pigment rather than from increase in amount. Others pointed out that sensitivity to ultraviolet radiation returns to normal before the tan disappears (Perthes, 1924; Schall and Alius, 1928,a,b).

The first to recognize the major factor contributing to the reduced sensitivity that develops after exposure to ultraviolet radiation was Guillaume. In a short paper in 1926 and in a book published in 1927 he called attention to the thickening of the corneum that results from the active epidermal proliferation which is one of the aspects of sunburn, and pointed out that this must greatly reduce the penetration of ultraviolet radiation to the malpighian layer. Histological studies by Lovisatti (1929) and by Miescher (1930) soon gave strong support to Guillaume's idea. As early as 24 hr after exposure to ultraviolet radiation, degenerative changes in the prickle cells of the malpighian layer are detectable. Later the whole layer including the basal cells may be involved, depending on the severity of the dose. When the acute stages of this reaction subside both the malpighian layer and the corneum are left thickened. An idea of the difference in penetration that this makes is illustrated in Fig. 13-7. The thickening is eventually reduced and in about two months may have returned to normal if there has been no further exposure to ultraviolet radiation. The erythema threshold returns to normal at about the same time.

As mentioned, vitiliginous areas of skin do not tan, but do increase their erythema thresholds after exposure to ultraviolet radiation (With, 1920; Meyer, 1924). The same is true for albino skin (Lovisatti, 1929). That decreased epidermal penetration resulting from thickening is the explanation in these cases is also supported by the decrease in transmission of the skin of the albino mouse after repeated exposure, which is illustrated in Fig. 13-7 (Kirby-Smith *et al.*, 1942; Blum and Kirby-Smith, 1942). In none of these cases is there any melanin pigment formed. Such evidence does not, of course, preclude the possibility that increase in melanin plays some part in reducing the amount of ultraviolet radiation reaching the malpighian layer, but this factor may be of only minor importance. It is easy to be misled by the observation that the skin becomes darker to the eye when tanning takes place, because this is not directly dependent on absorption by melanin within the erythema spectrum. Presumably, absorption in the latter region would be due largely to the phenolic ring, as in the case of protein. It seems doubtful that the increase of absorption due to melanin could be nearly as great as that due to increase of the protein layer by thickening of the corneum, and that the total attenuation of the radiation should be influenced to a much greater extent by the latter.



Of particular interest in this regard is Negro skin. Here melanin is not only more plentiful but is normally present in large amounts in the corneum rather than being concentrated in the neighborhood of the basal cell layer as in the unexposed skin of the white races. The Negro characteristically has a high erythema threshold (Miescher, 1932), and it might seem obvious to attribute this to the greater amount of melanin. However, it is generally agreed that the Negro corneum is considerably thicker than that of the white races—although no extensive histological account seems to have been published—and so the high erythema threshold may be due in this case to the protection afforded by a thick corneum rather than to high melanin content.

Because, as a rule, suntan and thickening of the corneum develop at about the same time, it is natural to associate the former with the protection afforded by the latter. Actually, however, the thickness of the corneum and the erythema threshold both return to normal in about two months if the skin is guarded from exposure to ultraviolet radiation. On the other hand, suntan may persist for months or years, fading away gradually, but at the same time subject to transient phases of bleaching and darkening. While as a general rule a tanned skin is less susceptible to sunburn than is an untanned one, this may be most misleading. There are persons who develop very little tan, yet can expose themselves to sunlight with relative impunity, and some who are fairly dark in complexion are comparatively sensitive to sunburn. In studying the erythema thresholds of a considerable number of subjects, the author has been unable to find any close correlation between complexion and sensitivity to ultraviolet radiation.

The possibility that the epidermal cells develop a local immunity, presumably by the formation of antibodies, was suggested by Perthes (1924); who carried out experiments which he interpreted as demonstrating such immunity, but these may have been confused by the inhibition of erythema (Blum and Terus, 1946a). The findings subsequent to Perthes' experiments indicate that it would be very difficult to demonstrate such immunity if it occurred. Hausmann and Spiegel-Adolph (1927) suggested that changes in the proteins of the corneum might lead to a decrease in transmission. The thickening of the corneum seems so obviously the major factor in limiting the amount of the erythema ultraviolet reaching the malpighian layer that other contributing factors would seem to be relatively unimportant.

*Artificial Protection.* During World War II the problem of evaluating various agents designed for protection of the skin against sunburn assumed certain minor importance. While the problem seemed at first relatively simple, it proved to be quite difficult, and no completely satisfactory solution was found. Only as the study progressed did its complexity become manifest, and in the end the most important thing gained was a somewhat

better understanding of the various factors involved. On first consideration the problem appears essentially a physical one. The desired end is to obtain a protective layer of some kind that will absorb the erythema spectrum but preferably not absorb much of the visible. Obviously, the film cannot be too thick, must be pliable, and should meet certain "cosmetic" requirements. The corneum of the skin, which may be taken as a model for it, seems to be a much better protective agent than any artificial one that has been devised. The corneum is a scattering and absorbing agent with a very high attenuation coefficient for the wave lengths of the erythema spectrum. The problem becomes more complex when another selectively absorbing layer is put on top of the corneum.

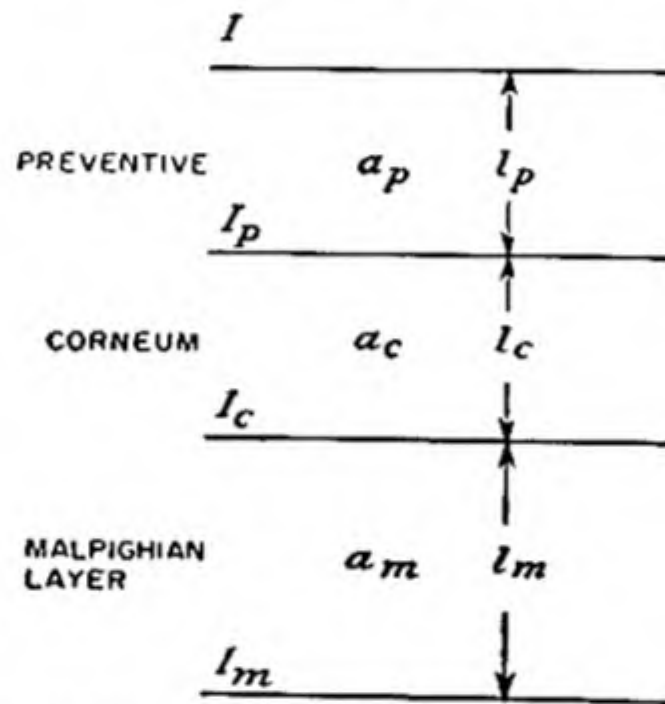


FIG. 13-9. Diagrammatic representation of the optical conditions of epidermis with a layer of preventive superposed.  $I$ ,  $I_p$ ,  $I_c$ , and  $I_m$  are intensities at the levels indicated;  $l_p$ ,  $l_c$ ,  $l_m$  are thicknesses of the layers indicated;  $a_p$ ,  $a_c$ , and  $a_m$  are attenuation coefficients of the layers indicated. (After Blum et al., 1946.)

We may extend our previous analysis to cover this situation. Figure 13-9, in which the preventive is indicated as having the thickness  $l_p$  and the attenuation coefficient  $a_p$ , illustrates this situation. The intensity  $I_p$  of a monochromatic beam at the bottom of this layer is given by

$$I_p = Ie^{-a_p l_p}. \quad (13-15)$$

Following the same reasoning as in developing Eqs. (13-9) to (13-12) we may write

$$Q_{p\lambda} = \frac{Dhc}{[e^{-a_p l_p} e^{-a_c l_c} (1 - e^{-a_m l_m}) \beta] \gamma \lambda} \quad (13-16)$$

to describe the situation when the preventive is in place. An index of the protection afforded by the preventive should be given by the ratio of the thresholds with ( $Q_{pr}$ ) and without ( $Q_r$ ) the preventive; this ratio we will designate for monochromatic radiation as  $P_\lambda$ , which is related to the other



quantities by

$$P_{\lambda} = \frac{Q_{pr}}{Q_{\tau}} = \frac{\frac{Dhc}{[e^{-a_p l_p} e^{-a_c l_c} (1 - e^{-a_m l_m}) \beta] \gamma \lambda}}{\frac{Dhc}{[e^{-a_c l_c} (1 - e^{-a_m l_m}) \beta] \gamma \lambda}}. \tag{13-17}$$

Since for monochromatic radiation the values of  $D$  at the threshold should be the same with or without the preventive, we may cancel out to obtain

$$P_{\lambda} = e^{-a_p l_p}. \tag{13-18}$$

That is, with monochromatic radiation the ratio  $P$  should measure, within the limits of accuracy, the attenuation by the preventive, and

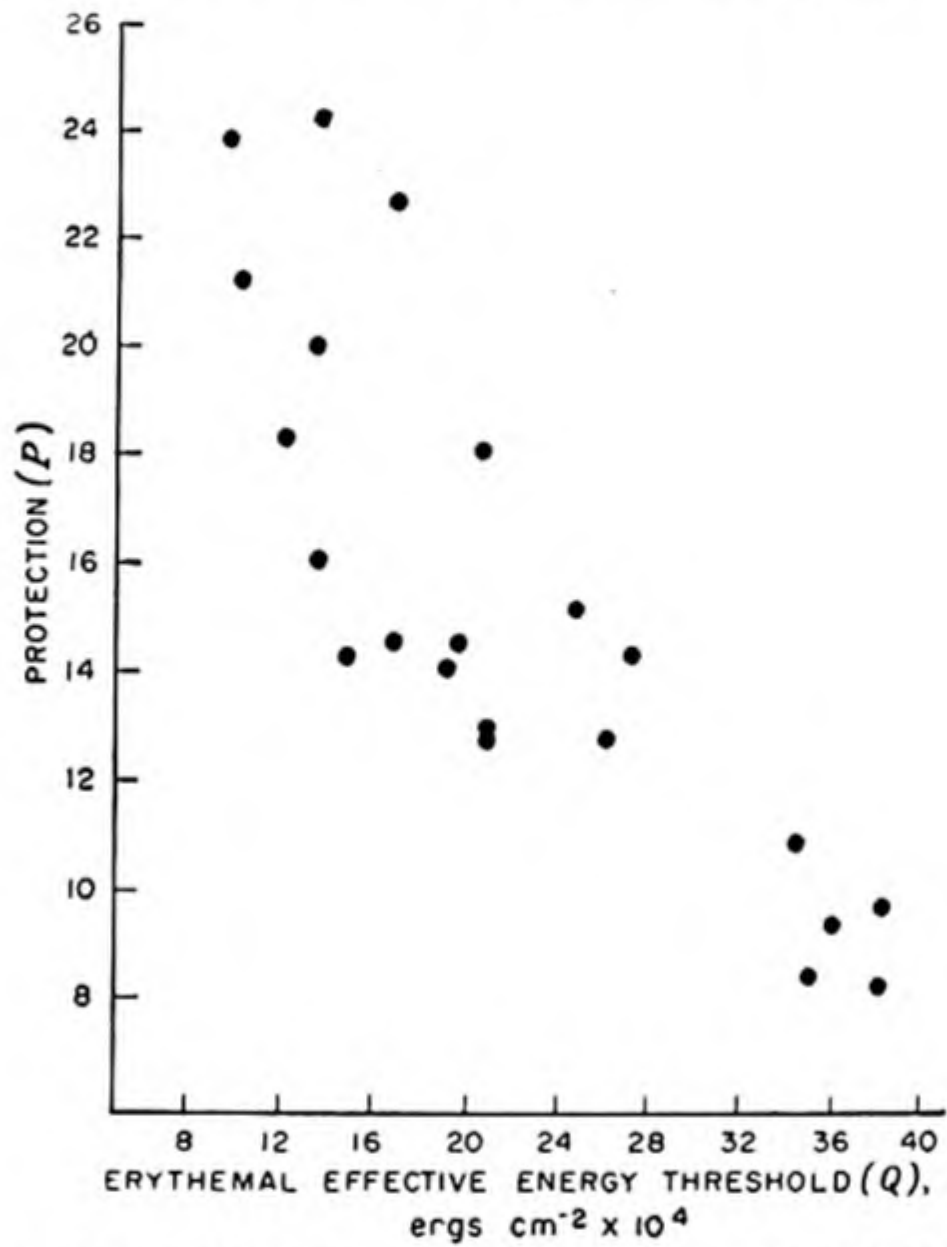


FIG. 13-10. Variation of protection  $P$  with erythemal threshold in 22 subjects. All the points were obtained with layers of the same sunburn preventive  $25\ \mu$  thick spread on skin of the abdomen. (After Blum et al., 1946.)

should be independent of the threshold of the individual.  $P_{\lambda}$  would, of course, vary with wave length according to the spectral characteristics of the preventive.

With polychromatic radiation—e.g., sunlight—the problem is not so simple, and in actual measurements it was found that the ratio  $P$  varied systematically with the threshold as illustrated in Fig. 13-10. Let us examine the possible reasons for such an unexpected variation.

We may write Eq. (13-13) in a more general form, such as might be used

for graphical integration when dealing with a spectrally continuous source such as sunlight within the limits  $u$ ,  $v$ , of the erythema spectrum

$$Q = hc \int_u^v \frac{Dhc d\lambda}{[e^{-a_{cl}}(1 - e^{-a_{ml}})\beta]\gamma\lambda} \quad (13-19)$$

We may write the corresponding equation from Eq. (13-16), when the preventive is in place, and describe the ratio of thresholds with and without the preventive as follows:

$$P = \frac{Q_{pr}}{Q_r} = \frac{hc \int_u^r \frac{Dhc d\lambda}{[e^{-a_{pl}}e^{-a_{cl}}(1 - e^{-a_{ml}})\beta]\gamma\lambda}}{hc \int_u^r \frac{Dhc d\lambda}{[e^{-a_{cl}}(1 - e^{-a_{ml}})\beta]\gamma\lambda}} \quad (13-20)$$

Quantities which could be canceled out in obtaining Eq. (13-18) now lie within the integral and cannot be canceled. Thus there must be uncertainty involved in the use of the ratio  $P$  as an index of the protection afforded against sunburn by sunlight or any other polychromatic source. This might account for a considerable amount of the variation found experimentally in the values of  $P$  obtained for different persons. The systematic variation with individual threshold, which is illustrated in Fig. 13-10, could be accounted for if we invoke the variation of  $D$  with wave length, for which the evidence has been discussed. If it is assumed that with no preventive the threshold is determined principally by the amount of absorption in the corneum, it may be assumed that in persons with high threshold a smaller proportion of the incident longer wave lengths of the erythema spectrum penetrate to the vessels of the dermis where such radiation inhibits the dilation of the minute vessels. Thus the inhibitory effect should be less in the person with a thick corneum who will also have a high threshold, tending to decrease the difference between the erythema threshold of high- and low-threshold persons. The same thickness of a given preventive should diminish the intensity of the radiation reaching the dermis in the same proportion in the case of the high- and the low-threshold persons, but would have a greater absolute effect on the latter than the former. This could account for the lower values of  $P$  found for high-threshold persons, which is illustrated in Fig. 13-10. On the other hand, if the threshold is considerably affected by the amount of dilator substance formed in the corneum, the picture may be changed somewhat.

The thing of practical importance is that the amount of protection measured by  $P$  varies with the threshold. Radiation from a carbon arc was used in making the measurements shown in Fig. 13-10, but the same systematic variation was found when similar measurements were made



with the mercury arc. Measurements made with the mercury arc gave much higher values of  $P$  than those obtained on the same persons with the carbon arc. The spectrum of neither of these sources closely resembles that of sunlight, and it is obvious that, with such wide variations in the obtainable values of  $P$ , tests of sunburn preventives with sources other than sunlight can have little practical significance as quantitative measures. Sunlight itself is a variable quantity, its intensity and spectral distribution changing radically with latitude, season, and time of day, and the validation of laboratory tests against actual field conditions is therefore beset with many unavoidable difficulties. Moreover, the difficulties of assembling an adequate number of human subjects under conditions appropriate for such validation may be overwhelming. It may be tempting to choose some simpler method without validation (see Giese and Wells, 1946a, b), but the dangers inherent in doing so must be obvious from what has just been stated. Eventually, physical methods such as that of Luckiesh *et al.* (1946) may replace the laborious testing on human skin, but these too will require validation (see Blum *et al.*, 1946, for further discussion of methods). It should be obvious that tests with methods chosen uncritically and without validation may be misleading.

### MECHANISMS

Up to this point sunburn has been treated as a complex physiological response further complicated by difficult optics. Only descriptions of various aspects of this complex have been given, with little attempt to explain the more intimate underlying mechanisms. It is now time to attempt to put together a general picture of the whole. In the scheme arranged in Fig. 13-11, it is represented that various physiological responses are mediated by specific substances elaborated as a result of the action of ultraviolet on living cells. In erythema this seems certainly to be the case, since wave lengths completely absorbed in the epidermis produce dilation of vessels in the underlying dermis, and such action seems to be interpretable only in terms of a transportable mediating substance. In other cases the presence of a mediating substance in the scheme can be considered as little more than a guess, and the tentativeness of the whole must be emphasized.

In the diagram (Fig. 13-11) the central theme evolves from an initiating photochemical reaction taking place in the malpighian layer, which results in the elaboration of a number of substances by the cells of that layer. Each of these substances induces a specific physiological response in the immediate locus of its elaboration or in a neighboring tissue. Consider first the nature of the initiating photochemical reaction in terms of the light-absorbing substance. As has been shown, the possibility of identifying this substance by means of the action spectrum is complicated by a

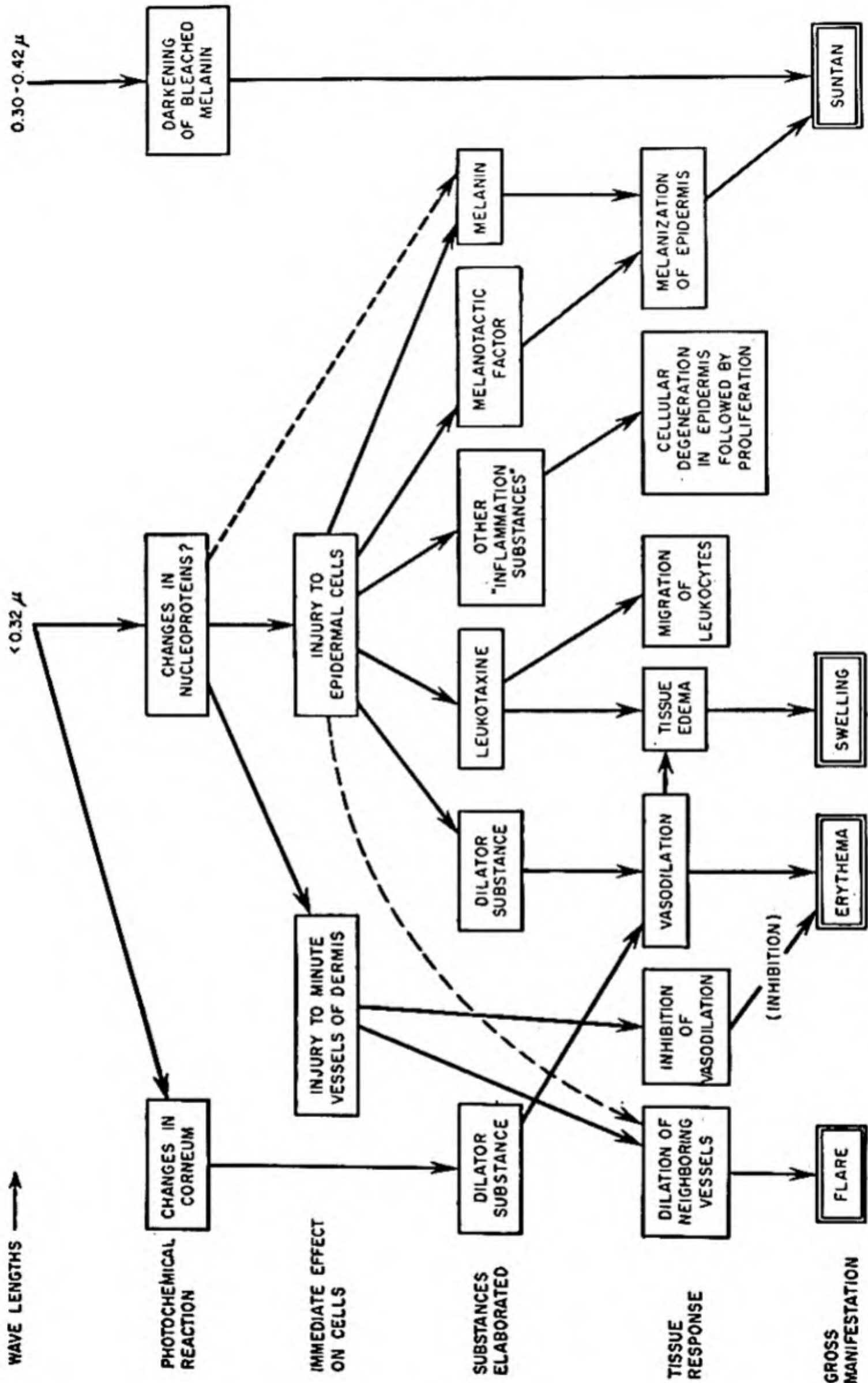


Fig. 13-11. Tentative scheme to describe the events of sunburn.



number of factors. The erythema spectrum is obviously dominated by the attenuating properties of the corneum which determine the spectral minimum at  $0.28 \mu$  and must influence the position of the maximum at  $0.297 \mu$ . Some of the attempts to interpret the erythema spectrum do not take this into account. The most adequate attempt to examine the erythema spectrum in terms of the radiation actually absorbed in the epidermis was that of Mitchell (1938) who used the data of Lucas (1931) to correct for epidermal transmission. Mitchell arrived at the conclusion that the light absorber is protein, but subsequent evidence throws doubt on this. In the first place, neither the inhibition of erythema by longer wave lengths nor the elaboration of dilator substance in the corneum was recognized at the time of Mitchell's analysis. These factors should affect the shape of the erythema spectrum to an unpredictable extent. It is implicit in Mitchell's analysis that the quantum efficiency [factor  $\gamma$  in Eq. (13-6)] is independent of wave length. But, if the inactivation of enzymes by ultraviolet radiation may be taken as characteristic of the action of this agent on proteins in general, this cannot be assumed since the inactivation is, in some cases at least, wave-length dependent (e.g., McLaren, 1947). The recent demonstration that dilator substance is formed in the corneum frustrates any attempt at analysis in terms of a single substance.

It seems altogether reasonable to draw an analogy between the effects of ultraviolet radiation on the cells of the human malpighian layer and the action of this agent on cells in general, which has been discussed elsewhere in this volume. Evidence seems at present to favor the idea that the light absorber in such effects is, as a rule, nucleic acid, and recent findings of Blum *et al.* (1950) indicate that, in the case of retardation of cell division in sea urchins' eggs, the locus of action is the nucleoprotein. But in these experiments, as is true in general, the ultraviolet radiation may possibly produce changes that are not reflected in the particular criterion studied (e.g., see Blum, Cook, and Loos, 1954). It must therefore be kept in mind that, although nucleoproteins in the malpighian cell are without doubt affected by the ultraviolet radiation that reaches these cells, this should also be true of the simple proteins in the cells to a greater or less extent. Thus, while it seems reasonable that the various physiological responses studied in sunburn are initiated by the action of ultraviolet radiation on nucleoproteins, it is possible that some of them involve other photochemical reactions. Other light absorbers have been suggested in connection with various aspects of sunburn (e.g., Ellinger, 1930; Rothman and Rubin, 1942; Frankenburger, 1933), but these have various objections which will not be discussed at length here (see Blum, 1941a, 1945). Even in systems optically much better suited to the comparison of action and absorption spectra, the distinction between nucleic acid and simple protein may be difficult, and there are other cell constituents absorbing in

the same region which, though present in much less amount, have rather similar absorption spectra. Thus attempts to analyze the erythema spectrum, even when carried out as carefully as Mitchell has done, cannot lead to very definite conclusions. So, while nucleoprotein has been indicated as the light absorber for the photochemical reaction initiating the principal theme schematized in Fig. 13-11, even this very basic postulate must be open to question (particularly in light of recent studies, Blum *et al.*, 1954).

In the diagram (Fig. 13-11) the immediate result of the photochemical reaction in the epidermal cells is described rather vaguely as injury. Among other substances elaborated by these cells is a dilator substance which brings about the vasodilation of the minute vessels of the dermis, and is grossly manifested as erythema. An increase in dilator substance has been demonstrated in skin (Nathan and Sack, 1922; Ellinger, 1930) and blood (Laurens and Kolnitz, 1940) of animals that have been exposed to ultraviolet radiation, but the identity of the dilator substance concerned in the erythema of sunburn has not been clearly shown. Lewis and Zotterman (1926) suggested that it was histamine or a histamine-like H-substance, and this view was once rather widely accepted. However, Percival and Scott (1931) found that the response of the skin to histamine pricks is not altered by the presence of the erythema of sunburn, and this is also true for skin which has been exposed to ultraviolet radiation but has not yet developed erythema (see Blum and Terus, 1946a). While the initial erythema of sunburn is rigidly restricted to the area exposed, after severe doses of ultraviolet radiation a red flare may develop which extends outward from the exposed area. This usually appears only after about 24 hr. The flare is irregular and differs in general appearance from the initial sharply limited erythema. Lewis and Zotterman (1926) called attention to the resemblance of this flare to that which results from pricking histamine into the skin, but, in the latter case, the flare appears almost immediately, and they do not adequately explain the delay in the case of sunburn. According to Lewis' hypothesis (1927), the histamine flare is the result of antidromic impulses in afferent nerves and in the case of sunburn it seems likely that stimulation of these nerves might result from injury to the fibers themselves or to surrounding tissues. The flare is most pronounced when the erythema is induced by longer wave lengths of the erythema spectrum, suggesting that the dermis itself, which is reached by those wave lengths, may be the locus of this effect. Krogh (1929) concluded that there must be at least two dilator substances concerned in sunburn, and, if erythema and flare are separate entities mediated by different substances, this should be true.

The whole question of the nature of the dilator substance is complicated by the finding that a dilator substance is produced by photochemical changes in the corneum (Rottier, 1952, 1953; Rottier and Mullink, 1952).



This has been included in the scheme without any commitment as to the nature of the light absorber or the dilator substance.

On the left of the scheme, injury to the minute vessels of the dermis is indicated as resulting in inhibition of their dilation. It may be supposed, in accordance with the argument already presented, that the underlying photochemical changes are essentially the same as those which result in erythema but that they occur at a different locus. It is suggested that injury to epidermal cells and to the vessels of the dermis may both be concerned in the dilation of vessels immediately adjacent to the area exposed to ultraviolet radiation, which manifests itself grossly as the delayed flare.

The various aspects of sunburn are essentially similar to those of inflammatory responses in general. Menkin (1940, 1942, 1943a, b, 1944) has succeeded in isolating from inflammatory exudates several substances which he concludes act specifically in bringing about certain of the tissue responses characteristic of inflammation. It seems not unlikely that the complicated picture presented by inflammation may ultimately be analyzed in terms of such "inflammation substances," and this general thesis has been followed in the scheme shown in Fig. 13-11 where the elaboration of a number of such substances is indicated as resulting from injury to the epidermal cells. According to Menkin, the increase in capillary permeability which occurs in inflammation is not due to histamine as supposed by Lewis and others but to a substance which he calls "leukotaxine." This substance also exerts a chemotactic effect, and, by virtue of both these actions, brings about the migration of the leukocytes from the capillaries into the surrounding tissues. In the present scheme leukotaxine is credited with bringing about the migration of leukocytes and with tissue edema, the latter being a direct result of increased capillary permeability. Indicated by a dotted line in the diagram is the probable participation of vasodilation as a lesser factor in the edema.

Cellular degeneration in the epidermis, followed by proliferation, is indicated as resulting directly from injury to the epidermal cells by the primary photochemical reaction. Alternatively, there is indicated the formation of other intermediary substances which may cause such cellular degeneration and proliferation. The substance necrosin, which Menkin (1943b) finds to cause the degeneration of cells, may be responsible in part for such changes in the epidermis and would be included in this category. It should be emphasized that none of these mediating substances has as yet been isolated from sunburned skin.

Consistent with the rest of the scheme, there has been introduced, as one of the products of the injury to epidermal cells, a "melanotactic" factor which contributes a part of the melanization of the epidermis that becomes manifest as suntan. This melanotactic factor presumably accounts for the migration of the melanin pigment which is observed in the early stage of the melanization process. The existence of such a sub-

stance is entirely hypothetical. Also indicated as a result of the injury to epidermal cells is the formation of new melanin which is the other factor contributing to melanization of the epidermis. A dotted line suggests the direct participation of ultraviolet radiation in the formation of melanin as a possible minor factor in melanization. Indicated on the extreme right of the diagram is the darkening of bleached melanin which is brought about by the longer wave lengths of the ultraviolet and the near-visible spectrum, approximately  $0.3\text{--}0.42\ \mu$ . This is an oxidation of bleached melanin by molecular oxygen as shown by Miescher and Minder (1939).

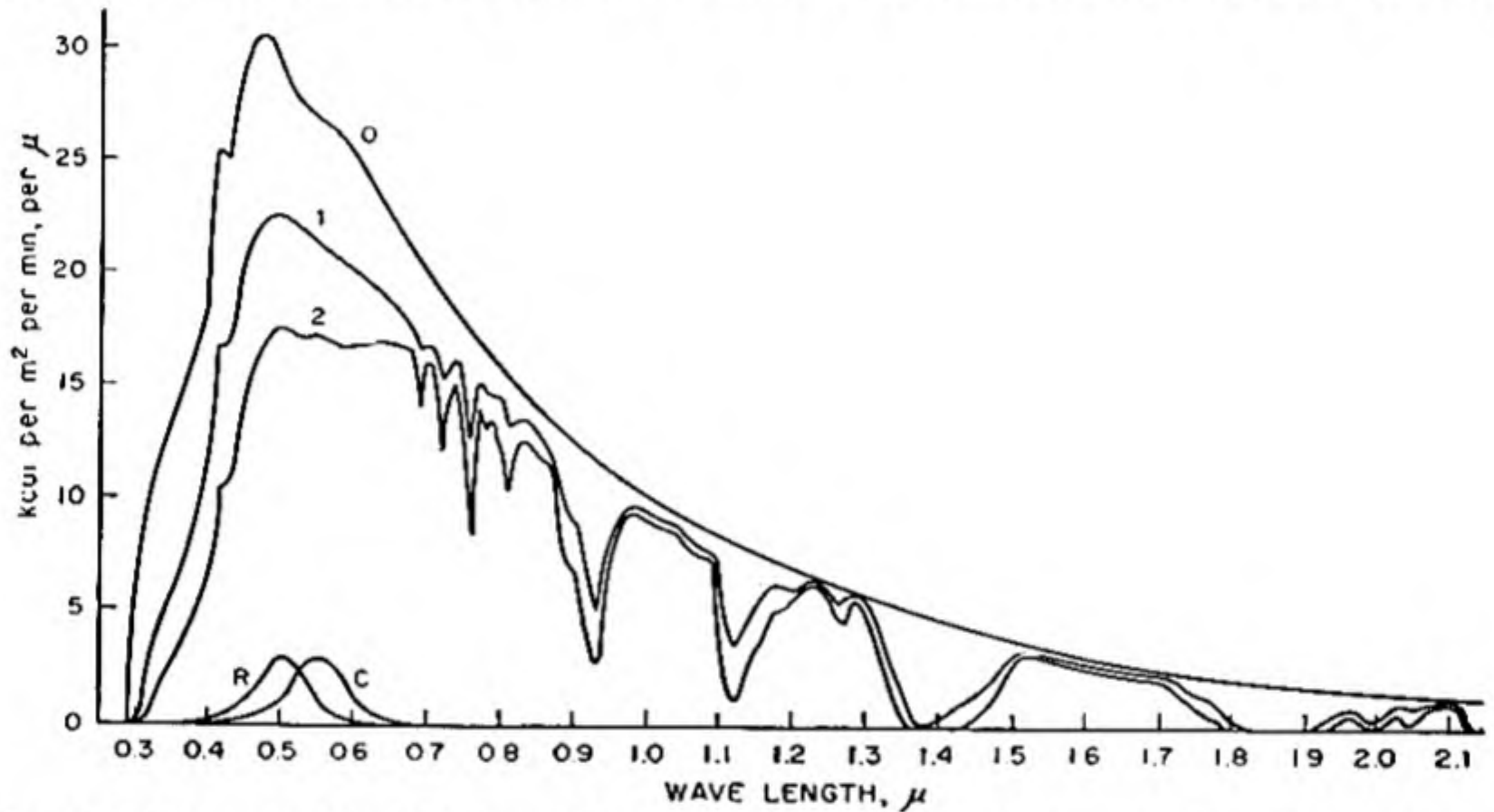


FIG. 13-12. Spectral distribution of sunlight. 0, outside the atmosphere (air mass 0); 1, with the sun at zenith (air mass 1); 2, with the sun  $60^\circ$  from zenith (air mass 2). Curves R and C indicate, respectively, the sensitivity of the human rods and cones; the ordinate units are arbitrarily chosen. (After Blum, 1945; data from Moon, 1941.)

Sunburn may logically be classed with other types of burn. There is a quantitative aspect to be recognized in that the primary damage in sunburn is very superficial because of the low penetration of ultraviolet radiation. As in the case of other superficial burns, some systemic involvement may be expected when the burned area is extensive enough. Studies of such systemic effects will not be discussed here, nor will the various claims for therapeutic effects of ultraviolet radiation. For an account of some of these, and of other effects of sunlight on man, the reader is referred to an earlier review (Blum, 1945).

*Sunburn by Natural Sunlight.* Many misconceptions about sunburn arise from failure to take into consideration the character of sunlight as an environmental factor. It is natural to evaluate the intensity of sunlight in terms of perception by the human eye, but this may be very misleading as regards the intensity of the sunburn-producing portion. The situation is best illustrated by referring to the diagram in Fig. 13-12, which shows



the spectral distribution of sunlight under different conditions. Curve 0 describes sunlight outside the earth's atmosphere as determined by extrapolation, showing a maximum at about  $0.48 \mu$ . As the sunlight passes through the atmosphere, its spectrum is modified by the absorption and scattering of some wave lengths to greater extent than others, as indicated in curves 1 and 2 of the figure. Curves R and C are, respectively, spectral sensitivity curves for the rods and cones of the human eye. It may be seen that the sensitivity of the eye becomes virtually nil before the long-wave-length limit of sunburn is reached at  $0.32 \mu$ . It is obvious, therefore, that the eye gives no direct information as to the amount of erythema radiation in sunlight. Neither does it give much indirect information, because some of the factors that greatly influence the intensity of the erythema portion of sunlight have little effect on the portion which the eye sees.

The amount of erythema radiation in sunlight varies much more widely with time of day, season, and other factors than does the visible portion of the spectrum. This is because of differences in absorption and scattering by the atmosphere. The intensity of the erythema region of the spectrum of sunlight is greatly diminished by absorption by ozone, which cuts off virtually all wave lengths shorter than  $0.285 \mu$ .<sup>19</sup> The wide variation in the intensity of the erythema portion of the spectrum with season, latitude, and time of day also depends on absorption by this gas. For example, when the sun moves from zenith to  $60^\circ$  from zenith—the conditions represented by curves 1 and 2—the erythema spectrum is greatly reduced. But the change in intensity registered by the human eye is relatively small because the gases of the atmosphere absorb the visible light only slightly. Sixty degrees in zenith angle corresponds to 4 hr in time, so even on a bright midsummer day one is not likely to be sunburned before 8 o'clock in the morning, or after 4 o'clock in the afternoon, although at both times the sun is shining brightly so far as the eye can discern. Obviously, chance of sunburn should be judged in terms of time of day rather than visual impression. Of course, it must be remembered to correct for daylight saving and for the difference between the two extremes of an official time zone, where the deviation from true time may be over an hour. Variation in ultraviolet radiation with season is another matter that tends to be left out of account. In the north temperate zone the maximum insolation occurs on June 21, the summer solstice. One unfamiliar with the problem may be surprised to learn that the erythema radiation may be more intense at the beginning of May than it is at the end of August. This is likely to lead to severe sunburn in the springtime when, moreover, the subject is not likely to have as thick a corneum as at

<sup>19</sup> For a discussion of solar radiation and the factors determining its intensity and spectral distribution at the earth's surface see Chap. 3 of this volume by Sanderson and Hulburt.

the end of the summer. There is a tendency to think of the tropics as receiving much more sunlight than the temperate zones and therefore to anticipate greater danger of sunburn in the tropics. This is again a question of season. At the time of the summer solstice the sun is over the Tropic of Cancer,  $23^{\circ}27'$  north of the equator. On that date there is about the same sunlight at  $47^{\circ}$  north—roughly the latitude of Seattle, Washington; St. John's, Newfoundland; and Paris, France—as at the equator. On June 21, other things being equal (the ozone layer probably would not be), there should be about as much danger of sunburning at the one latitude as at the other.

Another less obvious factor likely to mislead the unwary is the high proportion of erythema radiation that is contributed by reflection from the sky. Sky radiation is sunlight that has been scattered, principally by the gas molecules of the atmosphere. The shorter wave lengths are scattered to a greater extent than the longer ones, and hence the sky radiation is richer in the erythema radiation than in visible radiation.<sup>20</sup> At noon on a very clear day in temperate latitudes, the sky radiation may constitute only 10 to 15 per cent of the visible component falling on a horizontal surface, whereas for wave lengths shorter than  $0.32\ \mu$ , the direct and sky radiation are about equal under the same conditions (Pettit, 1932; Luckiesh *et al.*, 1944). Obviously one need not be directly exposed to the sun to receive a sunburn if he is sufficiently exposed to the sky. This explains why, for instance, one may sit under a beach umbrella protected from the direct rays of the sun and yet receive a severe sunburn from sky radiation which the umbrella does not cut off. On a lightly overcast day, particularly in a fog, the scattered erythema radiation may be many times the direct radiation and may cause a severe sunburn. At high latitudes at midday the sunburn-producing component of the sky radiation is also greater relative to that of the radiation coming directly from the sun than it is at low latitudes<sup>21</sup> (Coblentz *et al.*, 1942).

Dust and smoke absorb the erythema wave lengths very strongly, and a slight haze that is barely perceptible to the eye may completely wipe out this part of the spectrum. Hence sunburn is more likely in rural regions than in the neighborhood of industrial cities. The seashore with an onshore wind carrying away all traces of smoke or dust may be a particularly favorable place for sunburning, as may be the high mountains. Snow and ice reflect the erythema spectrum to a high degree, explaining in part at least the coining of the terms "snow burn" and "glacier burn." Water reflects less than is commonly believed (Coblentz *et al.*, 1933).

Although sunburn is primarily produced by wave lengths at the short

<sup>20</sup> The sky is blue for the same reason, blue and violet being scattered to a greater extent than the longer wave lengths.

<sup>21</sup> DeLong (1884) reported in the log of the ill-fated *Jeanette* the difficulties from sunburn during the arctic summer.



end of the solar spectrum—shorter than  $0.32\ \mu$ —it should not be forgotten that pigment darkening is brought about by somewhat longer wave lengths extending into the visible at about  $0.42\ \mu$ . Henschke and Schultze (1939b) showed that when suntan is produced by natural sunlight a good deal of the color may be due to pigment darkening rather than the production of new pigment, because of the high intensity of the wave lengths between  $0.32$  and  $0.42\ \mu$  as compared to those shorter than  $0.32\ \mu$  (see Fig. 13-8). This should be particularly true when the sun is far from zenith as in the later afternoon and early morning, and at noon in late fall or early spring in temperate latitudes. Differences in the pigment-darkening effect may explain why some persons retain a rather dark suntan throughout the winter months while others do not. In the early spring, when there is relatively little of the erythema radiation in sunlight but a considerable amount of the pigment-darkening radiation, darkening of the skin by the latter may be mistaken for new suntan. The belief that this darkening is accompanied by immunity to sunburn may lead to over-exposure when the erythema radiation increases with the progress of the season.

#### FACT AND FANCY

The convincing fact that one has been severely sunburned when he thought he was adequately protected from exposure looms large against any explanation that may be offered in terms of such a complex of factors as has been discussed. In the past, when still less was known about the subject, it was a natural tendency to invent factors—sometimes with little respect for physics or physiology—which would seem to explain such puzzling occurrences. In a book published in 1905 by Giles it is suggested that there are penetrating “Y” and “Z” rays in sunlight which are analogous to X rays and which account for the alleged injurious effects of tropical sunlight. While we may smile today at this naïve invention, we may wonder how much influence this book had in generating popular fear of tropical sunlight and in establishing such practices as the wearing of red spine pads in the tropics, which continued at least up to the time of World War II.<sup>22</sup> Another wholly uncritical book reflecting this fear was that of Woodruff (1905), on which Jack London based his diagnosis of his own breakdown during the *Cruise of the Snark*.<sup>23</sup> I recall too, what difficulty I had only a few years ago in convincing a practitioner of medicine that the “actinic” rays of the sun would not pass through the metal roof of an automobile. Certainly some of the difficulty one encounters in this respect comes from failure to recognize the importance of indirect radiation from the sky.

<sup>22</sup> An amusing account of field tests in the Philippines to determine the value of orange-red underwear (Phalen, 1910) shows how seriously such ideas were taken at one time in some quarters.

<sup>23</sup> The problem of tropical sunlight has been discussed elsewhere (Blum, 1945).

There is a widely accepted opinion that wet or perspiring skin is more susceptible to sunburn than dry skin. This was investigated by Blum and Terus (1946b), but no significant difference was found in the erythema threshold for skin wet with water from that for dry skin. Similarly, subjects showed no significant difference in threshold before and after profuse sweating in a "hot" room. I find a clue to the origin of this particular idea in a personal experience. A number of years ago I received a severe sunburn after an excursion in a small boat, during the whole of which I thought myself protected by a white shirt I was wearing. There were intermittent showers with bright sunlight between, and the sea was choppy so that my shirt and skin were drenched during most of the day, either from rain or spray. I remember that some additional protection was offered by my undershirt which was also wet but under which the sunburn was less severe. I might have concluded that wetting the skin had lowered the erythema threshold so that I sunburned through the shirt which I knew would have afforded adequate protection under other circumstances. The alternative answer was, of course, that the transmission of the erythema radiation by the shirt was increased by wetting. Support for the latter idea came some years later from tests of the protection afforded against sunburn by fabrics (Blum and Terus, 1946b), when it was found that various kinds of white shirtings became much more transparent to the erythema radiation when wet, presumably as a result of diminished scattering by the wet fibers.

The wide differences in individual erythema thresholds, the variation of the threshold in a given individual, and the failure to evaluate correctly the amount of exposure to erythema radiation in sunlight, are factors which no doubt contribute to the variety of opinions encountered regarding the efficiency of a given sunburn preventive. These factors, and the misconceptions regarding tanning, may account for many apparent vagaries of sunburn. In these pages I hope I have explained away a number of false ideas, but I have also taken the risk of introducing a few more by some of my speculations regarding the mechanism of sunburn (particularly in the scheme in Fig. 13-11). So, in ending, I should call attention to the need for further study of the sunburn process, always with proper regard for the known physical and physiological aspects of the problem lest we be led further into the realm of fancy.

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### ADDENDUM

Recent experiments in this laboratory indicate that the erythema threshold, primary pigmentation, and thickening of the epidermis, do not exhibit photorecovery. That is, illumination with "visible" light after exposure to ultraviolet does not affect these manifestations of sunburn.

## CHAPTER 14

### Ultraviolet Radiation and Cancer

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" . . . sans être héliophobe à outrance, je crois opportun d'arrêter l'attention de ceux qui abusent des bains de soleil sans aucun contrôle."

—A. H. Roffo

*Experimental studies: The carcinogenic wave lengths—Tumor types and penetration of the radiation—Quantitative aspects. Theoretical. The role of sunlight in cancer of the skin of man: Topographical distribution—Complexion—Occupation—Distribution. Prevention. References.*

In 1928, G. M. Findlay of Edinburgh published in *The Lancet* a brief paper describing the production of cancer in the skin of mice by repeated exposure to mercury arc radiation. He had been interested in the mechanism by which crude tar induces cancer, and this observation was more or less accidental. Within the next few years, three other groups of workers announced similar results. Putschar and Holtz (1930) in Göttingen and Roffo (1933) in Buenos Aires used rats as the experimental animals; Herlitz *et al.* (1931) in Stockholm used mice. It appears that none of these three groups was aware, when beginning its experiments, of the others' activity or of Findlay's previous paper, so all their results may be regarded as independent findings. Only Findlay and Roffo were directly interested in the problem of cancer, the latter undertaking the experiments because of ideas regarding the role of cholesterol in carcinogenesis. Herlitz *et al.* and Putschar and Holtz were interested in the effects of excessive dosage of ultraviolet on vitamin D, and did not anticipate the induction of cancers. Within the next decade a number of other workers carried out this type of experiment, and it became recognized

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generally that ultraviolet radiation is a carcinogenic agent for mice and rats.

These experimental studies revived interest in the idea, already over thirty years old at the time, that sunlight is a cause of cancer of human skin. The earliest to suggest this were Unna (1894), Dubreuilh (1896), and Sheild (1899). The most extensive observations were those of Dubreuilh on the workers in the vineyards of the Bordeaux region, among whom he found more cutaneous cancer than among the urban population. He called particular attention to the limitation of skin cancer to the face and hands, remarking that the position on the face seemed to accord with the area exposed by the peasant headdress. His studies were reported at length in 1907. There were also extensive reviews by Hyde in 1906 and by Bellini in 1909. Four principal lines of evidence supporting the idea that sunlight is a cause of cancer of the skin of man were based on these early clinical observations: (1) cancer of the skin occurs principally on parts exposed to sunlight; (2) cancer of the skin is more prevalent in outdoor workers than in sedentary workers; (3) the incidence of cancer of the skin is greater in regions of the earth that receive the greatest insolation; and (4) cancer of the skin occurs more often in light-complexioned persons than in dark. At the time these arguments were outlined, basic information that was essential to support them was lacking, and this information could not be supplied until experimental studies on animals had been initiated. The various lines of evidence will be examined in some detail in this chapter.

The laboratory studies have also provided a tool for studying the process of carcinogenesis by ultraviolet radiation. The successes and difficulties encountered will be discussed a little later. But the experiments themselves, what they show, and what their limitations are must be discussed before either the mechanism of carcinogenesis or the etiology of human cutaneous cancer is considered.

### EXPERIMENTAL STUDIES

*The Carcinogenic Wave Lengths.* Obviously, one of the first things to be done in the laboratory was to delimit the wave lengths that induce the cancers. The first attempt to do this was made by the late A. H. Roffo, whose findings appear extensively and rather diffusely described in a bulletin published in Spanish from his institute in Buenos Aires. Many of the more important ones are, however, briefly summarized in an article published in French in 1934. Roffo exposed rats to mercury arc radiation passing through various colored glasses. These filters are not accurately described, but one which is designated as "verre transparent" may be assumed to be common window glass. Whereas rats exposed directly to the mercury arc radiation developed cancers, those protected by this glass,

or by any of the colored ones, did not. In addition, Roffo carried out similar experiments with sunlight; he is, in fact, the only experimenter to have induced cancers with natural sunlight. In this case, too, "verre transparent" filters prevented the formation of tumors. If the assumption is correct that Roffo's "verre transparent" was ordinary window glass, he may be credited with having discovered that the carcinogenic wave lengths are those shorter than about  $0.32 \mu$ ,<sup>3</sup> i.e., that the carcinogenic ultraviolet has the same long-wave-length limit as the erythema spectrum. The question was definitely settled a little later. Funding *et al.* of Copenhagen in 1936 reported extensive experiments on the carcinogenic wave lengths before the Comité International de la Lumière at Wiesbaden. They found that a filter which transmitted wave lengths  $0.28 \mu$  and longer if placed in front of a mercury arc permitted induction of cancer, but that window glass cutting off the  $0.313 \mu$  and all lines of shorter wave length prevented induction of the tumors.<sup>4</sup> In 1941, Rusch *et al.* of Wisconsin reported similar experiments with like results. They were unaware of the earlier work of Funding *et al.*, which was not readily available. The experimental evidence serving to establish the long-wave-length limit of the carcinogenic radiation is summarized in Table 14-1, which includes a few additional observations.

Up to the present, no studies have been reported in which monochromatic radiation was used to induce cancer, other than some by Rusch *et al.* (1941) that seem inconclusive and some by Blum and Lippincott (1942) who used the  $0.2537\text{-}\mu$  line from a low-pressure mercury arc and showed that this wave length is weakly effective in inducing cancer in albino mice (see Table 14-1). The idea seems current that studies with monochromatic radiation should show much more than is already known about the carcinogenic process, and that studies with polychromatic radiation are faulty in some way not clearly specified. The difficulties of working with monochromatic radiation should become obvious when it is realized that groups of identically treated mice as large as 40 in number are needed if reasonably good comparisons are to be made. The difficulty of exposing such numbers of animals to monochromatic radiation must be obvious. But, besides the experimental infeasibility, there are apparently insurmountable difficulties of interpretation that would arise once the studies with monochromatic radiation were completed. An analysis by the writer (1943a), based on a few experiments with filters, indicates the uncertainty and the apparent futility of more exact measurements with monochromatic radiation. The situation may be compared with that of sunburn, discussed in Chap. 13. The erythema spectrum has been determined with monochromatic radiation, and a smooth curve may be drawn to describe it, whereas we have only a rough idea of the shape of

<sup>3</sup> Approximately the shortest wave lengths transmitted by ordinary window glass.

<sup>4</sup> See Fig. 13-3, Chap. 13, for the position of the mercury lines.



TABLE 14-1. EXPERIMENTS DETERMINING THE CARCINOGENIC WAVE LENGTHS  
(From Blum, 1948.)

Source	Investigators	Year	Animal	Filter	Shortest wave length reaching skin in appreciable amount, $\mu$	Tumor induction
Mercury arc <sup>a</sup> in quartz (intermediate pressure)	Roffo	1934	Rat Rat	None Window glass <sup>c</sup>	0.2302 <sup>b</sup> 0.3341 <sup>b</sup>	+ —
	Funding <i>et al.</i>	1936	Mouse	None S&G BG3 Window glass S&G BG8	0.2302 <sup>b</sup> 0.2820 <sup>d</sup> 0.3341 <sup>d</sup> 0.4108 <sup>d</sup>	+ + — —
	Rusch <i>et al.</i>	1941	Mouse	None Corning 970 Window glass Corning 352	0.2302 <sup>b</sup> 0.2925 <sup>d</sup> 0.3341 <sup>d</sup> 0.4046 <sup>d</sup>	+ + — —
	Blum	1943a	Mouse	None Pyrex glass Corex D Window glass <sup>e</sup>	0.2302 0.2967 0.2699 0.3341	+ + + —
	Bain and Rusch	1943	Mouse	Special	0.2925 <sup>d</sup>	+
Mercury arc <sup>f</sup> (low pressure)	Rusch <i>et al.</i>	1941	Mouse	None	0.2537 <sup>g</sup>	—
	Blum and Lippincott	1942	Mouse	None	0.2537	+
Sunlight	Roffo	1934	Rat Rat	None Window glass <sup>c</sup>	0.2900 0.3200 <sup>b</sup>	+ —
Tungsten filament	Findlay	1928	Rat	None	0.4000 <sup>b</sup>	—
	Roffo	1934	Rat	None	0.4000 <sup>b</sup>	—
	Rusch <i>et al.</i>	1941	Mouse	None	0.4000 <sup>b</sup>	—
SI <sup>h</sup>	Beard <i>et al.</i>	1936	Rat		0.2699 <sup>b</sup>	+

<sup>a</sup> The emission of such lamps is similar to that illustrated in Fig. 13-3. Other papers demonstrating carcinogenesis by such arcs are not included since they provide no further evidence regarding wave-length dependence.

<sup>b</sup> Based on the present author's estimate. The characteristics of the source as well as the transmission of the filter have been considered in making these estimates.

<sup>c</sup> "Verre transparent," which is assumed to be ordinary window glass, prevented carcinogenesis as did also several colored glasses which should have longer wave length cutoffs.

<sup>d</sup> Based on the original author's statement. The characteristics of the source as well as the transmission of the filter have been considered in making these estimates.

<sup>e</sup> Unpublished result.

<sup>f</sup> Nearly all the radiation emitted within the carcinogenic range is in the 2537 A line.

<sup>g</sup> The dosage used was apparently inadequate (compare with Blum and Lippincott, 1942).

<sup>h</sup> This is a combination of a tungsten filament and a mercury arc in special glass envelope.

the carcinogenic spectrum. The erythematous mechanism involves at least two light-absorbing substances, whereas the carcinogenesis process probably involves only one since reciprocity is obeyed with polychromatic light.

*Tumor Types and Penetration of the Radiation.* Those cutaneous tumors of man in which sunlight may have an etiological role are carcinomas of either the squamous cell or basal cell type. All originate in the epidermis. When experimental tumors were produced in rodents with ultraviolet radiation, it was generally expected that they would be of these same types. Indeed, the earlier reports indicated that this was generally true. Only Roffo (1934) claimed that a considerable proportion of the tumors induced by ultraviolet radiation were sarcomas, i.e., tumors of tissues underlying the epidermis. There remained a certain skepticism regarding this point when in 1940 experimental studies were initiated at The National Cancer Institute, but it was then found that the incidence of sarcomas induced in the ears of albino mice by ultraviolet radiation was very high—over 90 per cent (Grady *et al.*, 1941, 1943).

This apparent disparity between human cutaneous cancers and the tumors experimentally induced in the skin of mice needed to be resolved. It was thought that the differences in distribution of tumor types in the two cases might be explained by difference of penetration of the ultraviolet radiation, since mouse epidermis is considerably thinner than human epidermis. In order to settle this question, the transmission of ultraviolet radiation was measured for mouse skin and for human epidermis (Kirby-Smith *et al.*, 1942). Figure 13-7, in the preceding chapter, compares the spectral transmissions of samples of mouse and of human epidermis. In the mouse, as in man, the transmission decreases markedly after repeated exposures to ultraviolet radiation because of thickening of the corneum, but mouse skin always remains more transparent to the carcinogenic wave lengths than does human skin, even when the latter has not been exposed to ultraviolet radiation. On the basis of these measurements of physical penetration of the carcinogenic agent, it is altogether plausible that, if ultraviolet radiation is the common carcinogenic agent, tumors of human skin should occur almost entirely in the epidermis, whereas in the mouse deeper tissues are involved.

Differences in response of tissues to the action of the ultraviolet radiation might also be expected, and there is evidence of such differences. Table 14-2 shows the incidence of tumor types obtained under different experimental conditions. The size of the dose had little influence on the distribution of tumor types, and there is not much evidence of any relation to the time from the first dose to the appearance of the tumor.<sup>5</sup> On the other hand, the ratio of carcinomas to sarcomas differed markedly with the frequency with which the doses were given. When the dose was

<sup>5</sup> The "development time" defined in the next section.



repeated daily, or 5 days a week, there was a relatively high percentage of carcinomas. When there was only one dose per week, sarcomas predominated. This difference obviously cannot be attributed to penetration of the radiation.

TABLE 14-2. DISTRIBUTION OF TYPES OF TUMOR INDUCED BY ULTRAVIOLET RADIATION IN THE EARS OF ALBINO MICE (STRAIN A)  
(After Grady *et al.*, 1943.)

		No. of animals	Tumor types		
			Carcinoma	Sarcoma	Carcinoma/ sarcoma ratio
Dose (5 exposures per week)	Weekly dose (ergs/cm <sup>2</sup> ):				
	43.0	15	5	15	0.333
	16.5	62	9	59	0.153
	13.0	95	27	90	0.300
	9.9	77	12	75	0.160
	7.9	70	20	67	0.298
	5.3	66	20	62	0.323
	3.6	76	19	72	0.264
		Total 461			
Individual induc- tion time, <i>t<sub>d</sub></i> (5 exposures per week)	<i>t<sub>d</sub></i> (days):				
	100-150	112	20	106	0.189
	150-200	186	45	177	0.254
	200-250	88	23	86	0.267
	250-300	42	12	40	0.300
	300 +	33	12	31	0.387
		Total 461			
Schedule of expo- sures	No. of expo- sures per week:				
	1	60	3	60	0.050
	5	461	112	440	0.255
	7	77	32	68	0.470
		Total 598			

In total, about 95 per cent of the tumors were pure sarcomas or sarcoma mixed with carcinoma. About 25 per cent of the tumors contained carcinoma, a few of these being pure carcinomas, but for the most part mixed with sarcoma.<sup>6</sup> The sarcomas were predominately spindle-cell types, most of them presumably arising from connective tissue, but some from muscular elements. There were a few hemangiomas, one sebaceous

<sup>6</sup> These figures are for tumors of the ear only, the eye and other sites not being included.

gland tumor, and one osteochondrosarcoma. The small number of carcinomas fits with the thinness of the malpighian layer in the mouse ear, which never becomes as thick as it does in human epidermis and hence never absorbs as large a proportion of the incident radiation. When the attempt is made to compare the incidence of occurrence of tumors or of the type of tumors induced by ultraviolet radiation in different species of animals, this question of penetration must be taken into account. An interesting experiment in this regard was made by Hueper (1941), who elected to study the effects of ultraviolet radiation on congenitally hairless rats with the expectation that he would obtain more tumors than with haired mice. To his surprise, only one of the hairless rats developed a tumor and this was a carcinoma. On examination he found that the corneum of the hairless rats was very much thicker than the corneum of the haired animals, which could account for his findings. The generally greater incidence of carcinomas in rats than in mice (Putschar and Holtz, 1930; Roffo, 1934; Beard *et al.*, 1936) may be explainable in similar terms.

The morphological character of these tumors leave little doubt as to their malignancy. Metastasis is relatively rare, but it does occur. This is also characteristic of most cutaneous tumors of man, the malignant melanomas being an outstanding exception. Transplantation has been successfully carried out in some instances. For a more complete discussion of tumor types and other aspects of their pathology, the original papers (Grady *et al.*, 1941, 1943) should be consulted.

*Quantitative Aspects.* In discussing the quantitative aspects of the induction of tumors by ultraviolet radiation reference is made principally to a series of studies carried on at The National Cancer Institute, since they provide the most extensive data collected thus far and since, because of the method used, they are quantitatively intercomparable. Precautions were taken in these experiments to assure that the data would be reproducible. In the first place a genetically homogeneous strain of mice, strain A, was used. This is an albino mouse which tends to develop tumors of the lung but which does not spontaneously develop cutaneous tumors. The females of this strain also develop cancer of the breast, but the males do not; therefore, in order to reduce the number of variables, only the latter sex was used. The diet and other treatment of the animals was made as uniform as feasible so as to reduce biological variability to a minimum.

The mice were subjected to measured doses of ultraviolet radiation from a mercury arc given at regular intervals. After a considerable number of doses—in only a very few cases was the dosage period less than 100 days—tumors appeared on the ears of the mice, the time of appearance depending on the size and the frequency of the dose. The time from the application of the first dose of ultraviolet radiation to the appearance of such a tumor in a given mouse is here called the “development time,” and in the fol-



lowing discussion is symbolized as  $t_d$ . For practical reasons, the appearance of a tumor was taken as the time at which a tumor of a given estimated size, approximately  $100 \text{ mm}^3$  in volume and consisting of about  $10^8$  cells, was present on the ear. At this time the tumors were usually doubling their volume every few days, so that the error in estimation of  $t_d$  was relatively small. Since the ears of these mice are almost hairless, the skin was exposed directly to the ultraviolet radiation. No tumors

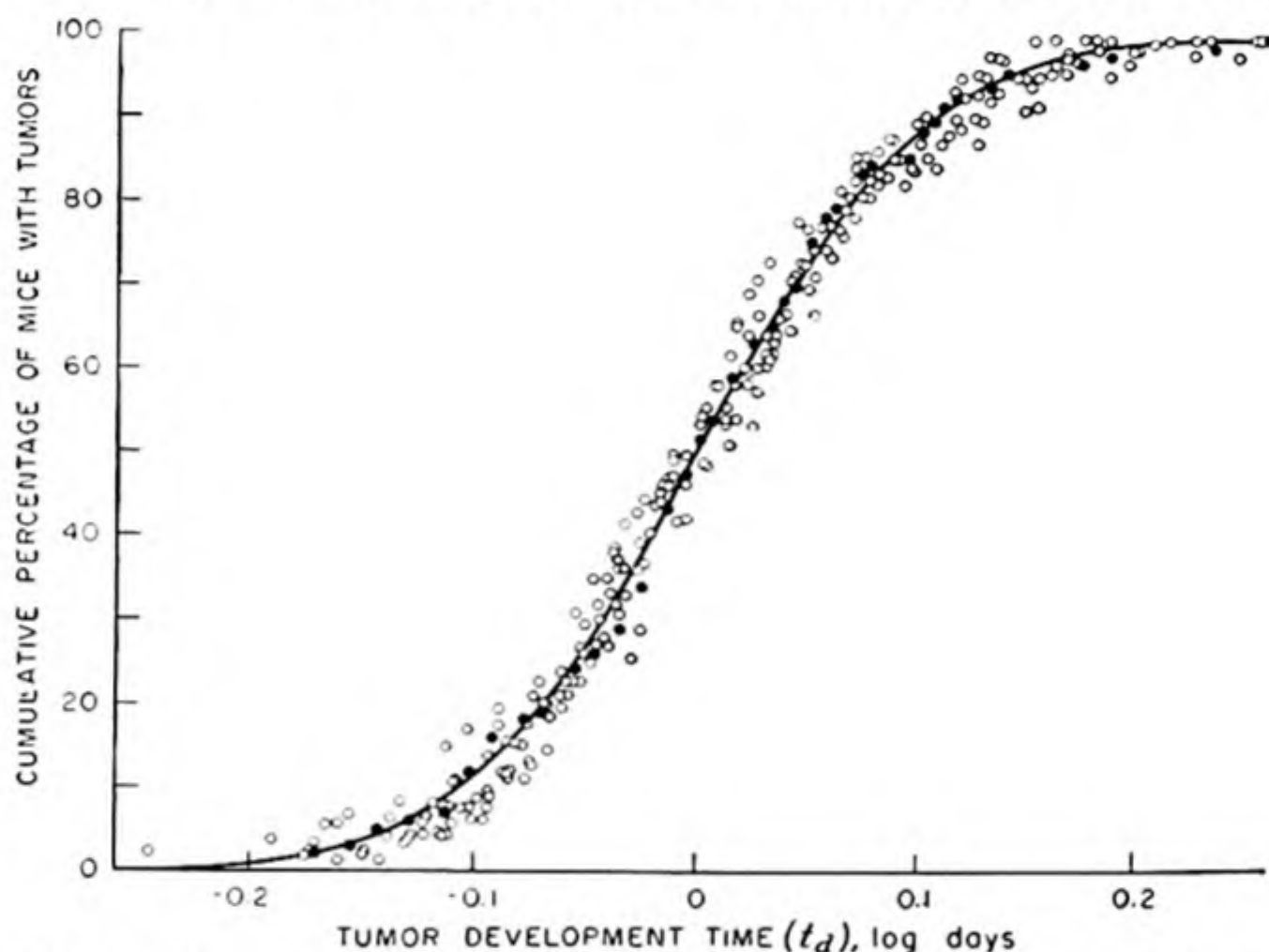


FIG. 14-1. Distribution of the logarithm of the tumor development time,  $t_d$ , in a population of genetically homogeneous (strain A) male mice, exposed to ultraviolet radiation at regular intervals up to the time of tumor appearance. The data are from 12 experiments, a total of 676 mice. The dose and interval between doses was the same for a given experiment but varied between experiments. The mean values of  $t_d$  varied accordingly, and in the figure all the points have been corrected to a common, mean value for each experiment, i.e.,  $\log t_d = 0$ . The smallest number of animals in any single experiment was 41, the largest 98. The solid dots represent the experiment with the 98 animals. The drawn curve is the integral of a normal distribution, with standard deviation 0.081 log days. The same distribution was found for a number of additional experiments in which smaller numbers of mice were used.

were observed to occur on the well-furred parts of the body; rarely, a tumor appeared on a paw, the snout, or the tail. Tumors of the eye appeared in about 10 per cent of the mice (Lippincott and Blum, 1943). Since it was important to deal with as nearly uniform an area of skin as possible, only tumors of the ear were considered in the data referred to subsequently.

When the same dose of radiation was applied at regular intervals until the tumor appeared, the distribution of  $t_d$  in the population followed a smooth quantitative relation. In Fig. 14-1 the cumulative percentage of mice with tumors is plotted against the logarithm of  $t_d$ , and it is seen

that the points scatter about a smooth S-shaped curve, which is the integral of a normal distribution. For different doses or different schedules of exposure, the curve changes its position along the time axis, but the shape and slope are not changed. In this figure, data from different experimental series involving different dosages and schedules are brought together to a common point at 50 per cent incidence of tumors. The

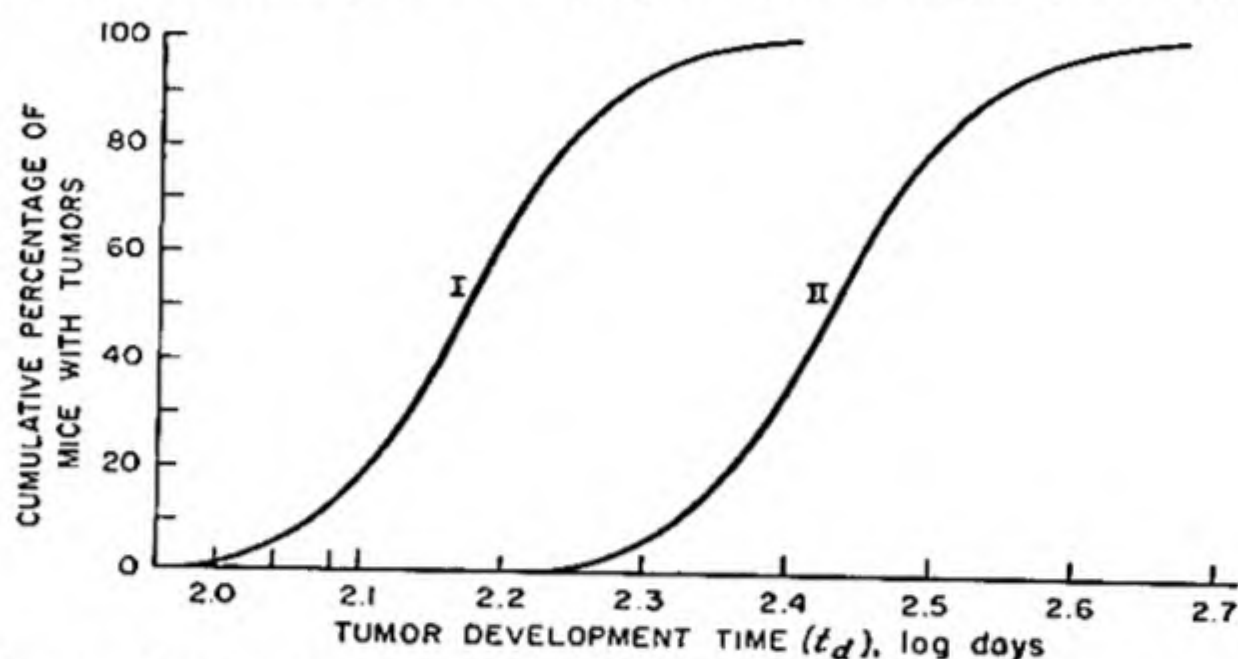


FIG. 14-2. Curves, for two experiments, of percentage incidence plotted against  $\log t_d$ . Curve II might represent a lower dose  $D$  or a longer interval between doses  $i$  than does curve I. Note that the shape and slope are the same for both experiments although the development time is longer for curve II than for I.

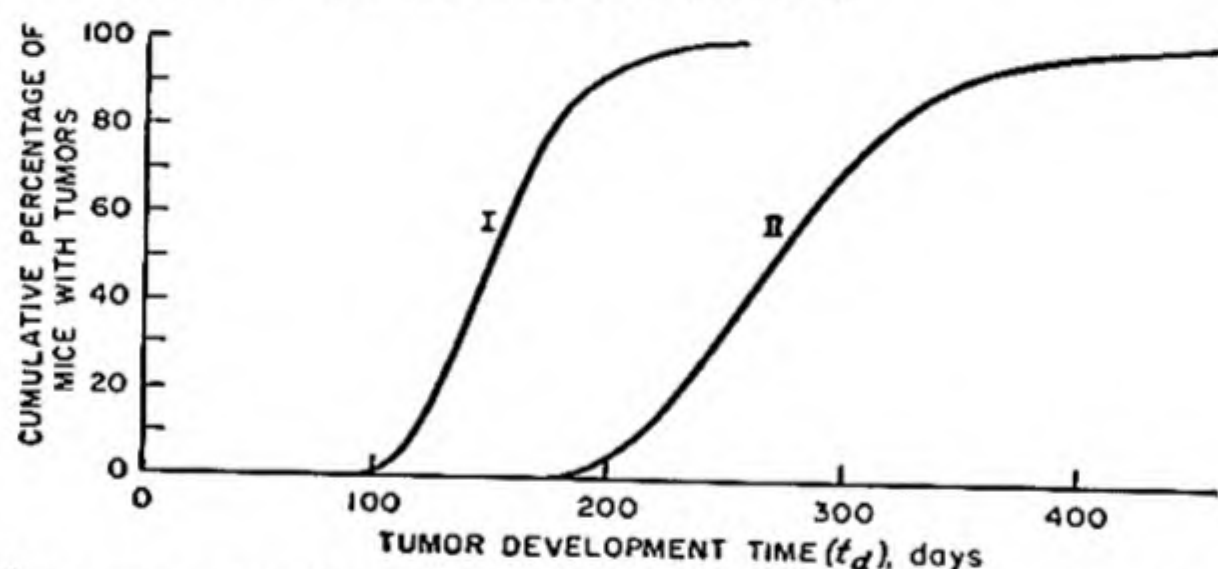


FIG. 14-3. The same percentage incidence curves represented in Fig. 14-2 but plotted against days instead of log days.

drawn curve represents a standard deviation of 0.081 log days, representing large variance in spite of all the precautions that were taken, although less than has been found for other types of carcinogenesis. The nice fit to a probability function makes it possible to assess the significance of the data statistically; and the uniform distribution of the points around the curve indicates the reliability of the data and the constancy of the shape and the slope of the curve. The success of the logarithmic plotting is indicated by comparing Fig. 14-2, in which the log of  $t_d$  is plotted, with Fig. 14-3, in which  $t_d$  is plotted directly.

To induce tumors it was necessary to repeat the dose many times. In



no case did a tumor develop as the result of a single dose. Change in the schedule of doses, for example, by discontinuing them after a given time, markedly delays the appearance of tumors and alters the shape of the curves, as is seen in Figs. 14-8 and 9.

Particular attention was paid to the reproducibility of the doses of ultraviolet radiation, since uniformity was necessary if the experiments were to have any quantitative significance.<sup>7</sup> The dose was customarily measured in units of  $10^7$  ergs/cm<sup>2</sup> of radiation of wave lengths  $0.313 \mu$  and shorter delivered by the particular type of intermediate arc used. The number of these units per dose is indicated in the following discussion by the symbol  $D$ . Experiments designed to test reciprocity showed that  $t_d$  is independent of intensity, at least down to a level well below that at which the data used in the following analysis were obtained.

### THEORETICAL<sup>8</sup>

Any theoretical treatment of carcinogenesis meets a difficulty that is seemingly insurmountable at the present time, namely, that a cancer is detectable only after it has reached a relatively advanced stage. An isolated cancer cell has the same general morphological characteristics as the normal cells of the tissue from which it arises. A cancer is recognized by the arrangement and behavior of these cells, and there must be present something like 1000 to 10,000 cancer cells before an observer can be sure that a cancer exists. The same is true of any chemical measurement, since changes are determinable only when a large number of cancer cells are already present.

Confronted with a cancer at such a relatively advanced stage, the observer may ask whether it has sprung into being suddenly or has grown for a considerable time before it was first detected. This question cannot be answered directly in the present stage of knowledge. Lacking any present means for detecting the original cancer cell, the observer can only extrapolate back from what he finds after the time of appearance of the tumor. Such extrapolation is certainly justified, but any reasoning based thereon must be considered as tentative and likely to be in error. When data are available such as those which have been obtained with ultraviolet-induced tumors—highly reproducible and exact within the limits of experimental error—the experimenter feels compelled to attempt to use it to learn something of the events prior to the appearance of the tumor if there is any chance that this can be done.

<sup>7</sup> The original paper (Blum *et al.*, 1941) should be consulted for such details as a description of the method of exposure of the animals and measurement of the dose.

<sup>8</sup> This analysis was recently presented in a paper which should be consulted for all details (Blum, 1950b). An extension of this analysis is now in course of preparation for publication.

Cancers grow as the result of the proliferation of cells; at least, this is what happens after they first appear. And so it must be assumed that the rate of tumor growth is always some function of the number of existing actively dividing cells. In general, the individual cancer cell does not enlarge beyond a certain quickly attained volume, and the rate of growth of a tumor is also a function of its volume. Hence the growth may be described by giving its volume  $V$ , or, alternatively, the number of cells composing it,  $N$ , as a function  $f$ , of the time  $t$ ,

$$\begin{aligned} V &= f(t) \\ Nv &= f(t) \end{aligned} \quad (14-1)$$

where  $v$  is the average volume of the individual tumor cell, assumed to be constant. An exact understanding of the process of tumor development requires knowing how this function depends on the conditions under which the tumor grows. Since the character of this function during the earlier stages of tumor development is not determinable at present, any hypothesis regarding carcinogenesis is in a quantitative sense an extrapolation.

For purely formal illustrative purposes let us begin by considering growth at a constant rate—the simplest possible type of growth. We assume that after somehow having reached a volume  $V_0$ , the tumor grows at a constant relative rate. We may think of  $V_0$  as the initial volume, i.e., the volume at zero time. The constant growth rate is expressed by

$$\frac{dV}{dt} = GV \quad (14-2)$$

where  $G$  is a constant. Integrating, we obtain

$$\ln \frac{V}{V_0} = Gt \quad (14-3)$$

in which  $V$  is the volume at the end of time  $t$ . Similarly, if it is assumed that a tumor is composed of a large number of cells and these are dividing at random with respect to each other but at a constant rate, the growth of the tumor is described by

$$\frac{dN}{dt} = G'N \quad (14-4)$$

and

$$\ln \frac{N}{N_0} = G't. \quad (14-5)$$

In the present case  $G'$  may be treated as equal to  $G$ .

Assuming such constancy of relative rate of tumor growth, we may examine the consequences. In the experiments described above the tumor has at time  $t_a$  a volume  $V_a$  of approximately 100 mm<sup>3</sup>. For pur-



poses of illustration  $t_d$  will be taken as 150 days, a value which is consistent with experiment. We may assume any given volume  $V_0$  for the tumor at the time the first dose of ultraviolet radiation is applied, and calculate the shape the growth curve would take throughout the course of the development of the tumor if the growth rate remained constant. This is done for values of  $V_0$  in Fig. 14-4a, using Eq. (14-3). In this figure curve I is based on an initial volume of  $10^{-6}$  mm<sup>3</sup>, the approximate volume of a single cell. On casual examination of this curve it seems that there is a long period of time during which nothing happens, but this is only because the volume during this period is too small to show on the graph. The volume at the

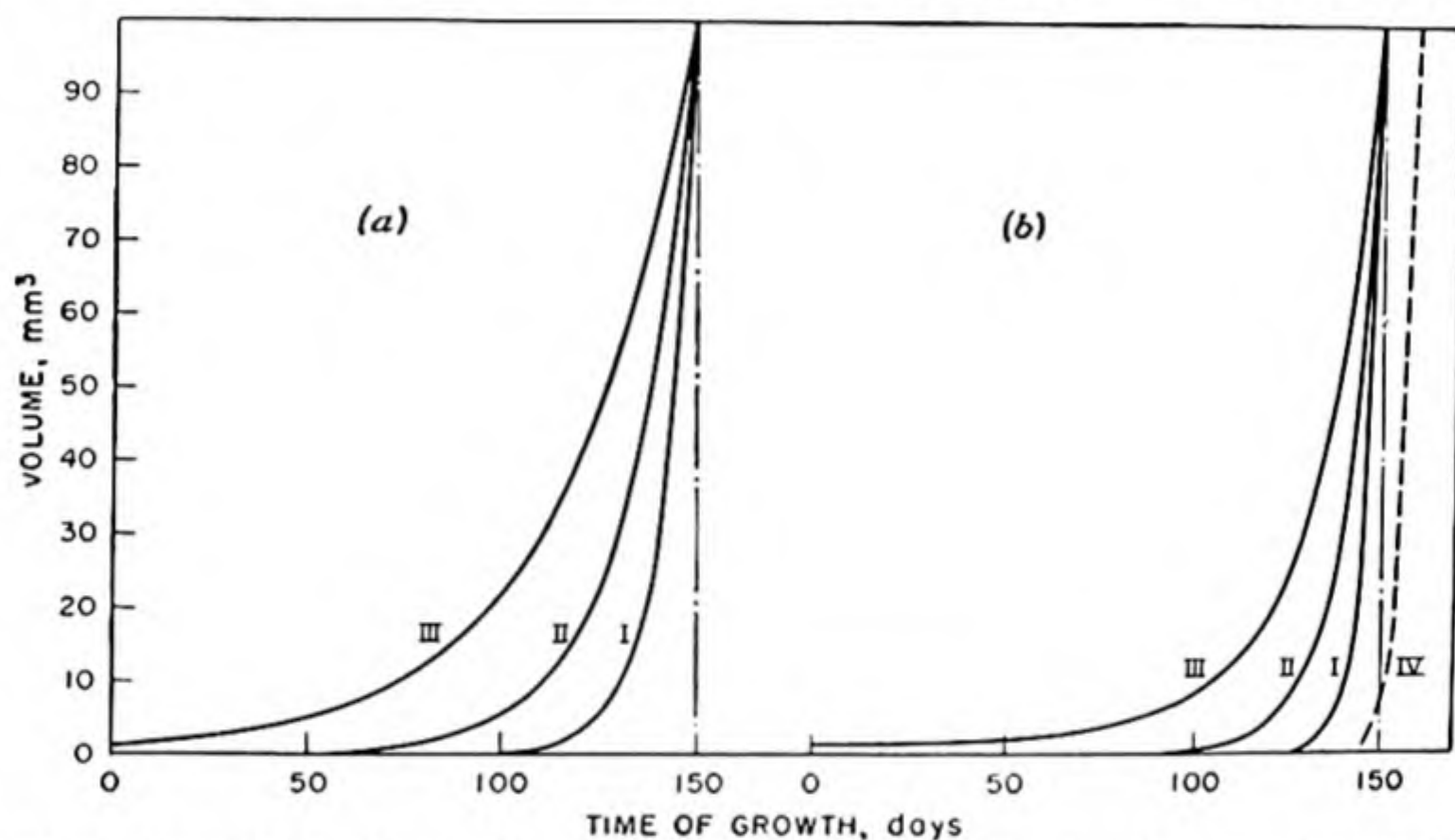


FIG. 14-4. Hypothetical growth curves. (a) Growth following the simple equation of relative growth. Curve I,  $V_0 = 10^{-6}$  mm<sup>3</sup>; II,  $V_0 = 10^{-2}$  mm<sup>3</sup>; III,  $V_0 = 1$  mm<sup>3</sup>. (b) Progressively accelerated growth curves. Curve I,  $V_0 = 10^{-6}$  mm<sup>3</sup>; II,  $V_0 = 10^{-2}$  mm<sup>3</sup>; III,  $V_0 = 1$  mm<sup>3</sup>; IV,  $V_0 = 10^{-6}$  mm<sup>3</sup> but rate slightly less than for curve I. (From Blum, 1950.)

end of 100 days is 0.2 mm<sup>3</sup>. A tumor of this size would be too small to be detected grossly in the living animal. Suppose, then, that a tumor originated as a single cell and grew at a constant rate. No tumor would be grossly observable until at least 100 days after the advent of the first tumor cell. We see at once that a gross fallacy is possible in those theories which assume that the period previous to the actual appearance of the tumor is occupied in the "induction" of the first tumor cells. The fact that a tumor appears quite suddenly at a time long after application of a carcinogenic agent cannot, without other evidence, be taken to mean that the tumor has not been growing ever since the first application of the agent. Curve II in Fig. 14-4a is based on the assumption that the tumor has a volume of  $10^{-2}$  mm<sup>3</sup> at the beginning of the curve, corresponding approximately to 10,000 tumor cells. In this case the tumor would not reach an observable volume until after 50 days. In the case of curve III,

a just observable volume,  $1 \text{ mm}^3$  is assumed at the beginning of  $t_d$ . The rates of growth are, of course, quite different for the three curves. That for curve I ( $G = 0.127$ ) is close to the average estimate for experimental tumors after they were large enough to be measured.

It seems hardly necessary to point out that the idea of growth of the tumor at a constant rate is incompatible with the experimental finding that tumors appear only if repeated doses are given during the time  $t_d$ , so the simple equation we have used cannot be applicable to our experimental data. It has been used only to illustrate the inherent weakness of the assumption, implicit in many theories of carcinogenesis, that separate induction and growth periods can be supported by purely qualitative observations. There are further objections in the present instance.

Hypotheses based on the idea of separate induction and growth periods usually contain the tacit assumption that the tumor cells proliferate in a more or less unrestricted fashion once they are formed. The tumor is often said to grow "autonomously," which seems to mean that the rate of proliferation of the tumor cells is inherent in these cells themselves. If this were so there should be no direct relation between their proliferation rate and the time required for tumor induction. To see how the present data bear on this point let us assume for purposes of argument that the development time may be separated into two distinct periods of induction and growth, which may be represented by the equation

$$t_d = t_i + t_g \quad (14-6)$$

where  $t_i$  represents an induction period during which the tumor cells are formed and  $t_g$  represents the growth period during which these tumor cells proliferate. Using the symbols from Eqs. (14-4) and (14-5) with similar meaning, we may write

$$t_g = f(N, G', \dots) \quad (14-7)$$

to indicate that the growth period is a function of the number of cells  $N$ , and of the rate of their proliferation which is related to  $G'$ . The function is indicated as incomplete because other unspecified factors may enter; thus we are not restricted to accepting the relative growth equation in its simple form. Combining the last two equations, we obtain

$$t_d = t_i + f(N, G', \dots). \quad (14-8)$$

Now we are confronted with a serious obstacle. Examination of Figs. 14-1 and 2 shows that the tumor incidence curves for a constant schedule of doses always have the same shape and slope when plotted against the logarithm of  $t_d$ . This can mean only that the shape and slope of the curves are continuously determined by some basic relation. To rationalize such a relation with Eqs. (14-6 to 14-8), the induction period  $t_i$  and the growth period  $t_g$  would always have to be proportional. It follows



that the induction period would have to be a function of both the number of tumor cells and the proliferation rate of these cells since these factors are going to determine the subsequent growth period. That is,  $t_i$  varies as  $f(N, G', \dots)$ . We see, therefore, that before the hypothesis assumed in setting up the first of these equations can be accepted, some mechanism must be found by which the induction period always adjusts itself so as to be proportional to the growth period that is going to follow it. Such a mechanism could hardly be consistent with the idea that the tumor cells proliferate autonomously at their own inherent rate, once they are formed, without reference to their past history, since the rate would have to be determined by the same factors that determine the length of the "induction" period. It does not appear that any one of the qualitative hypotheses assuming separate induction and growth periods provides such a mechanism.

Let us now examine another attractive and popular idea of carcinogenesis—that cancer cells are somatic mutants—to see how this fits with these data. The somatic mutation theory of cancer was formulated in the early part of this century and has been variously applied (see Strong, 1949). It met with little favor for a long time but of recent years has been rather widely accepted. This theory postulates that tumor cells originate from normal tissue cells by mutation, thus assuming characteristics which distinguish the tumor cells from normal cells. The idea might have particular interest with regard to the induction of tumors by ultraviolet radiation since this agent causes mutations in a multiplicity of living organisms. The theory implies the ostensibly irreversible change of a normal tissue cell into a cancer cell. The cancer cell has new characteristics which it transmits to all its daughter cells. One of the outstanding characteristics of all tumor cells is that, in some stages at least, they proliferate more rapidly than their fellows, and, since this aspect may be treated quantitatively, our attention may be focused upon it to the exclusion of others. The same kind of quantitative relations might be expected for any change inherited in the same manner. Let us examine these relations in terms of our data. One of the facts clearly shown by the experiments is that repeated doses of ultraviolet radiation are required to cause a tumor to appear within the lifetime of the mouse. In terms of the mutation hypothesis, this might be interpreted to mean that a given cell must be repeatedly acted upon by the radiation before it mutates; but this seems improbable since typical mutations, whether induced by ultraviolet or some other agent, occur suddenly. A more definite objection is that, as has been shown, the idea of an induction period followed by a period of unrestricted growth is not compatible with the data.

On first consideration it seems that these objections might be avoided. We may imagine that with each dose of ultraviolet radiation applied to the skin of the mouse, a corresponding number of normal cells mutate to

tumor cells. If it is assumed that the number of tumor cells so produced is small and their inherent rate of proliferation low, it is conceivable that no tumor would be detected, within the lifetime of the mouse, as a result of a single or a small number of doses of ultraviolet radiation. On the other hand, if the dose is repeated many times at appropriate intervals, it might be that enough tumor cells would be produced by mutation and by proliferation of the mutants to result in an observable tumor. Such a concept fits *qualitatively* with the experimental finding that a single dose of ultraviolet radiation does not produce a tumor, but repeated doses do.

When this concept is examined *quantitatively*, however, grave difficulties arise, as a simple arithmetical calculation shows. This is illustrated in Table 14-3, where it is assumed that each tumor cell divides every 6 days;

TABLE 14-3. GROWTH OF A TUMOR: ASSUMING THAT ONE TUMOR CELL ORIGINATES EACH DAY BY MUTATION AND THAT ALL TUMOR CELLS DIVIDE EVERY SIXTH DAY

Time from first exposure, days	Total cells from mutation	Total cells from proliferation	Total cells in tumor
0	1	0	1
6	7	1	8
12	13	16	22
18	19	44	50
24	25	100	106
30	31	212	218
36	37	436	442
42	43	884	890
48	49	1780	1786
54	55	3572	3578

this corresponds to the average growth rate for these tumors after they have reached measurable size. It was also assumed that one dose of ultraviolet radiation is applied each day, and that this dose causes one tissue cell to mutate to a tumor cell. Within a relatively short time after the first dose the contribution of tumor cells by mutation becomes negligible compared to the number contributed by proliferation of those tumor cells already present. Substitution of other reasonable values for the ones used in the example leads to essentially the same result. It is obvious that, if the development of a tumor depended on the accumulation of tumor cells by mutation and proliferation under conditions such as those suggested, after a very short time the application of further doses of radiation would have no detectable effect on the rate of development of tumors. Yet the application of doses of radiation as late as, say, 100 days after the first dose may have a marked effect on the rate of development of the tumors, as is illustrated in Fig. 14-8. It can only be concluded that the rate of tumor growth is not constant throughout the



development time and that the idea that ultraviolet radiation acts by forming tumor cells which immediately adopt a new and thenceforward constant rate of growth is not consistent with the experimental findings.<sup>9</sup> While this may not constitute an absolute negation of the idea of somatic mutation, it is evident that the hypothesis must be radically modified if it is to fit the experimental data. It may be seen that these quantitative experiments on the induction of cancer by ultraviolet radiation pose serious difficulties to theories of carcinogenesis which have been quite widely accepted by various groups of workers.

Another tentative theory will now be described, which is treated elsewhere in more detail than can be afforded in this chapter (Blum, 1950b). The theory is a quantitatively descriptive one which does not attempt

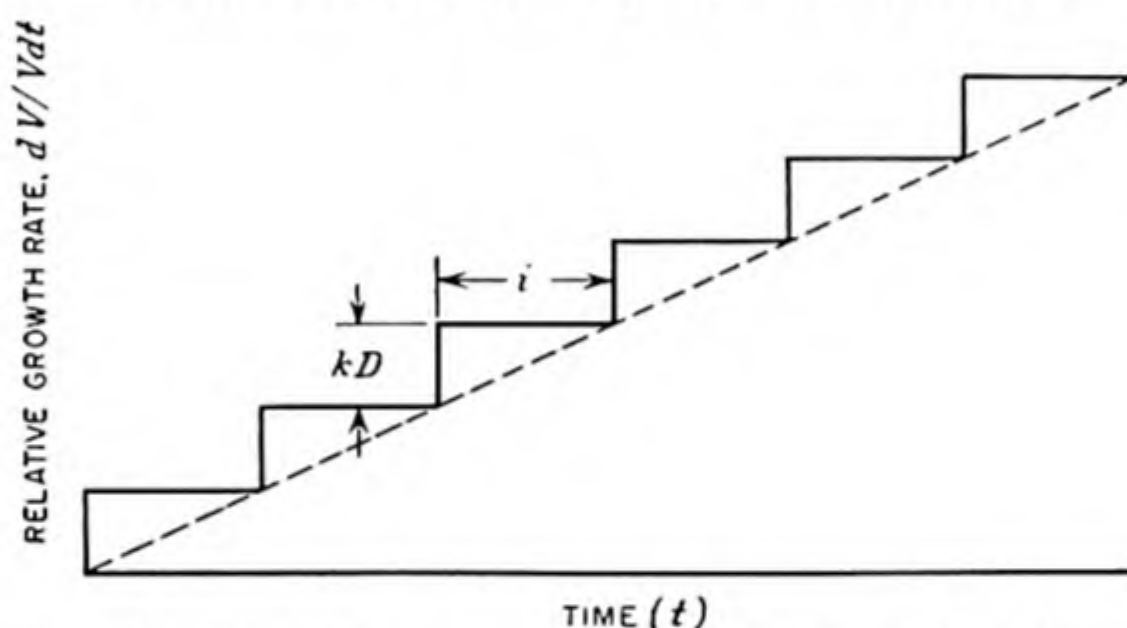


FIG. 14-5. Diagram to illustrate progressive acceleration of relative growth rates.

thus far to postulate any intimate mechanism for carcinogenesis, but suggests a frame within which such a mechanism needs to be fitted.

The theory is based on the postulate that successive doses of radiation progressively accelerate the relative rate of cell proliferation, each dose bringing about an increase in rate. In formulating an approximate model to describe this progressive acceleration of growth rate, we begin by rewriting Eq. (14-2):

$$\frac{dV}{V dt} = G. \quad (14-9)$$

The left-hand member of this equation represents the relative instantaneous growth rate, which can be plotted against time, as in Fig. 14-5. If the rate remained constant, i.e., if Eq. (14-9) were obeyed strictly, the line representing the growth rate would be parallel to the abscissa. Let it now be assumed that, on receiving each dose of ultraviolet radiation, the tumor grows at a rate which is increased by an amount roughly proportional to the dose, where the factor of proportionality may depend on the past history of the course of exposures but does not change rapidly from

<sup>9</sup> This criticism appears to extend to the hypothesis of Iversen and Arley (1953), who seem to have overlooked our argument.

day to day. This is illustrated in Fig. 14-5, where the growth rate is shown as rising abruptly to a new level with each dose, the new rate being maintained until the next dose is received. Without assuming that this diagram pictures the exact happenings in a tumor, it may be accepted as an approximation which may be put to test.

The dotted line in the figure represents a smooth acceleration, which for a long series of doses would very closely approximate the stepwise curve we have drawn. This line is described by

$$\frac{dV}{V dt} = \frac{kDt}{i} \quad (14-10)$$

which, when  $D$  and  $i$  are constant, may be integrated to

$$\ln \frac{V}{V_0} = \frac{kDt^2}{2i} \quad (14-11)$$

where  $V_0$  and  $V$  are the volumes of the tumor at the beginning and end of the time  $t$ ,  $D$  is the dose of ultraviolet radiation,  $i$  is the interval between successive doses, and  $k$  is a proportionality constant.<sup>10</sup> The rate of growth is assumed to be negligible at the time of the first dose.

The development time  $t_d$  has already been defined as the time from the first exposure until the tumor reaches a given volume designated as  $V_d$ . For this case, Eq. (14-11) may be rewritten as

$$\ln \frac{V_d}{V_0} = \frac{kDt_d^2}{2i} \quad (14-12)$$

If, in addition, the assumption is made that  $V_0$  is the same in all cases, this is equivalent to saying that  $V_d/V_0$  is a constant at the time  $t_d$ .<sup>11</sup> For any series of experiments in which the interval  $i$  is maintained constant,  $t_d$  should therefore vary inversely as the square root of the dose  $D$ , since by rearrangement we obtain

$$t_d = \left[ \frac{2i}{k} \ln \frac{V_d}{V_0} \right]^{1/2} D^{-1/2} \quad (14-13)$$

and all the values within the brackets are constants. Figure 14-6 illustrates what happens when this relation is applied to the data. In this figure values of  $t_d$  are plotted against  $D$ , on log-log coordinates. The values of  $t_d$  plotted are based on the time to 50 per cent incidence of tumors within groups of identically treated mice. Examination of the

<sup>10</sup> The equation may be used to describe tumor growth when  $t$  has high values, but does not hold exactly for short periods. A treatment applicable to the latter which has to be used in interpreting some of the data is developed more completely in the original paper, but Eq. (14-11) serves the present purpose.

<sup>11</sup> This is a tentative assumption. The data are better fitted if  $V_0$  is considered to vary (unpublished analysis).



curves in Figs. 14-1 and 2 will show that this is a justifiable procedure since, because of the nature of the incidence curves, the same relation holds for any other percentage incidence. In 11 of the experiments described in Fig. 14-6 the doses were applied 5 days per week, the interval being taken as  $7/5$  days and the curve so labeled in the figure. Above a certain value of  $D$ , designated  $D_m$ ,  $t_d$  does not decrease with increase in  $D$ , i.e., the curve is a horizontal line. Below  $D_m$  the points are quite well fitted by the drawn curve which has the slope  $-\frac{1}{2}$ , representing the condition, explicit in Eq. (14-13), that  $t_d$  varies inversely as the square root of the dose. The reason for the flattening of the curve at  $D_m$  will be discussed later. Figure 14-6 also shows data for dose intervals of 1 day and

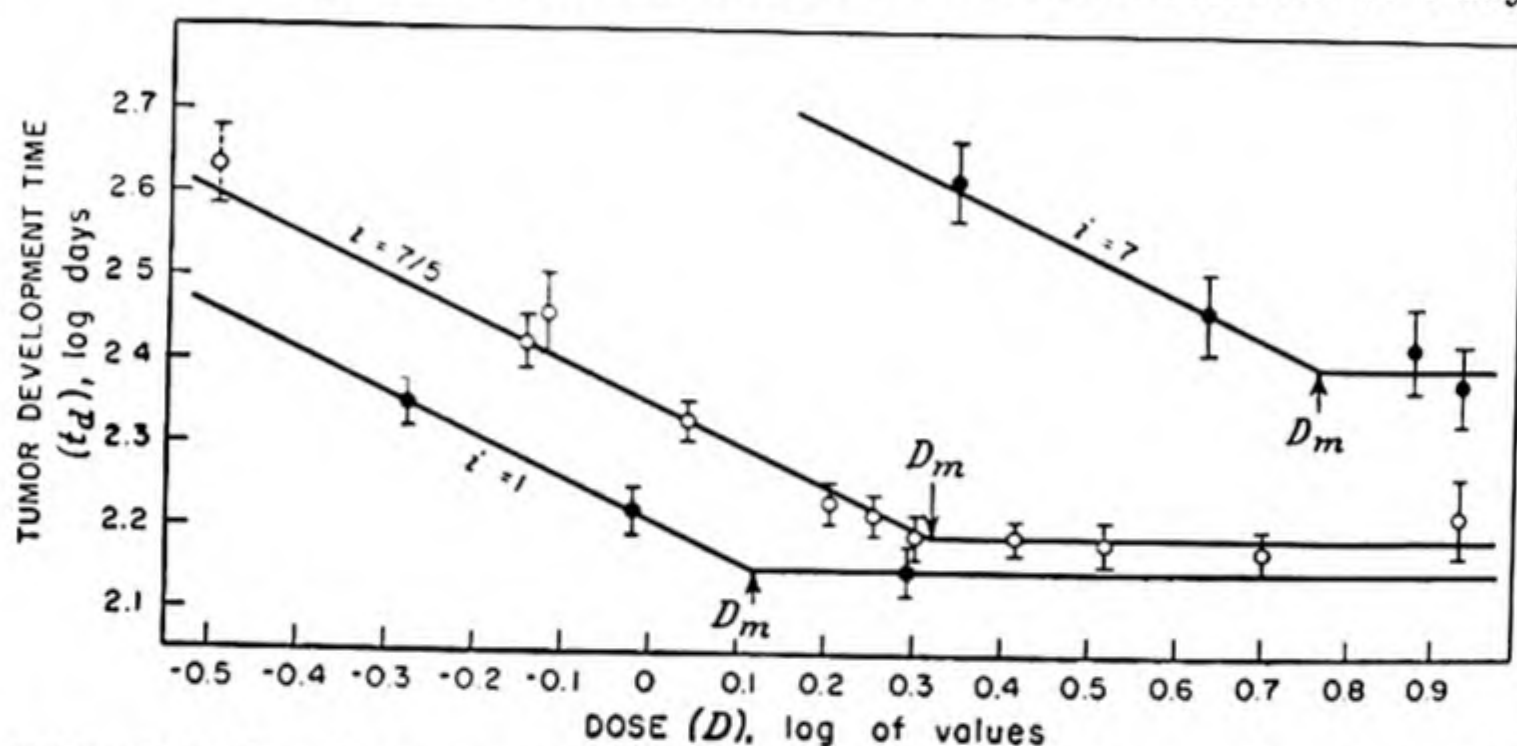


FIG. 14-6. Relation between dose  $D$  and development time  $t_d$  for three different intervals  $i$  (days). The relative accuracy of the points is indicated by the vertical lines, which represent the limits which should be exceeded only once in 20 times on the basis of chance alone. The symbol  $D_m$  indicates the point beyond which  $t_d$  does not decrease with increase in dose. (From Blum, 1950.)

of 7 days. The drawn curves resemble the curve for the 5-day-per-week schedule. There are insufficient points on the curves for the 1- and 7-day intervals to establish the shape assigned, but the agreement with the curve of the 5-day-per-week schedule is obvious. The fit of the equation to the data is also shown in Fig. 14-7, where the curves are plotted on numerical ordinates. The graphs in Figs. 14-6 and 7 show that the data support the theory within the limits of accuracy as well as could be expected.

Equations (14-12) and (14-13) do not hold exactly for different values of  $i$ , necessitating the following correction:

$$\ln \frac{V_d}{V_0} = \frac{kDt_d^2}{2(i-a)}, \quad (14-14)$$

where  $a$  is a constant having the value 0.52. This modification does not, however, affect the general argument.

Most of the tumors grow rapidly once they appear. In some cases

they double in volume in as little as 2 or 3 days. Curves illustrating certain aspects of progressively accelerated growth are shown in Fig. 14-4b. Curve I is an accelerated growth curve following Eq. (14-12) in which  $V_d/V_0$  is taken as  $10^8$  and  $t_d$  as 150 days. The value  $10^8$  represents the change from a single cell of volume  $10^{-6}$  mm<sup>3</sup> to a tumor of 100 mm<sup>3</sup>, that is,  $V_d$ . As the curve shows, such a tumor would first be grossly detectable about 20 days before it reached 100 mm<sup>3</sup> volume. This agrees in general with observation, since, as a rule, these tumors are not grossly manifest for more than two or three weeks before they reach this volume.

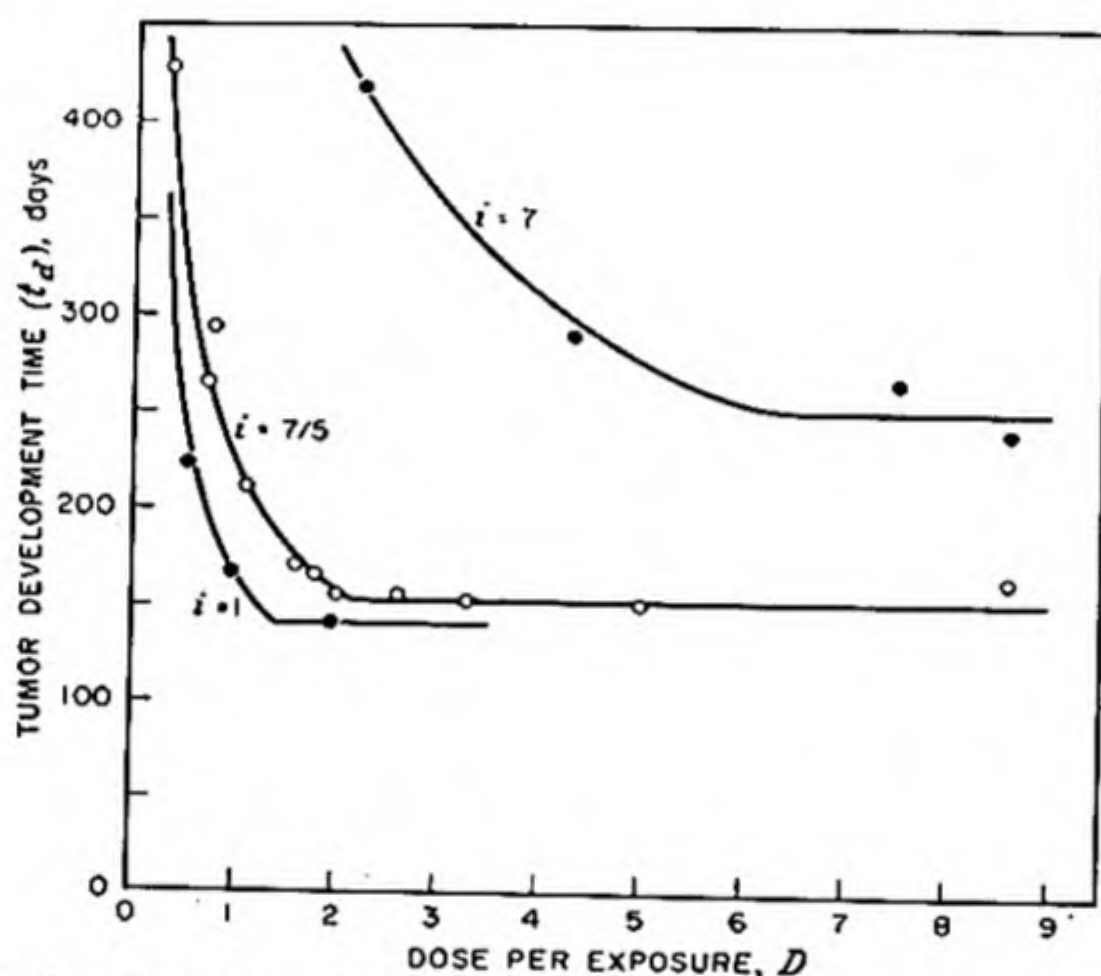


FIG. 14-7. Relation between dose  $D$  and development time  $t_d$  for three different intervals  $i$ . The drawn curves follow the theory of progressive acceleration of tumor growth discussed in the text. The data and curves are the same as those plotted in a different manner in Fig. 14-6. (From Blum, 1950.)

To illustrate another aspect of the problem, let us assume for purposes of discussion that every tumor develops from foci of cells, the growth of which foci is progressively accelerated by successive doses of radiation. Let us imagine that there are two foci, one growing thus at a "fast" rate and the other at a "slow" rate. If the growth rate of the fast focus exceeds that of the slow focus by only a very little, the ultimate tumor would arise almost exclusively from the fast focus. As shown in Fig. 14-4b, the broken curve IV is based on the same value of  $V_d/V_0$  as is curve I, but is calculated for a slightly lower acceleration. At 150 days, when curve I arrives at volume 100 mm<sup>3</sup>, curve IV is only about 10 per cent as high, yet curve IV if continued reaches 100 mm<sup>3</sup> only 10 days later than I. The slow focus represented by curve IV would contribute only 10 per cent to the total tumor volume at time  $t_d$ . If it is assumed that there is some



sort of distribution of the rates of growth of the tumor cells, only those growing near the maximum rate would contribute appreciably to the total tumor volume. If there were many foci of tumor cells in close proximity, those which proliferated most rapidly might so far cutstrip the others that the tumor when finally formed would be composed almost entirely of cells arising from fast-growing foci.

We have not yet accounted for the maximum limit of growth rate represented in the curves by the failure of increase in dose to decrease the time to appearance of tumors after a certain value  $D_m$  is reached (see Figs. 14-6 and 7). It must be obvious that there is a maximum rate of growth which

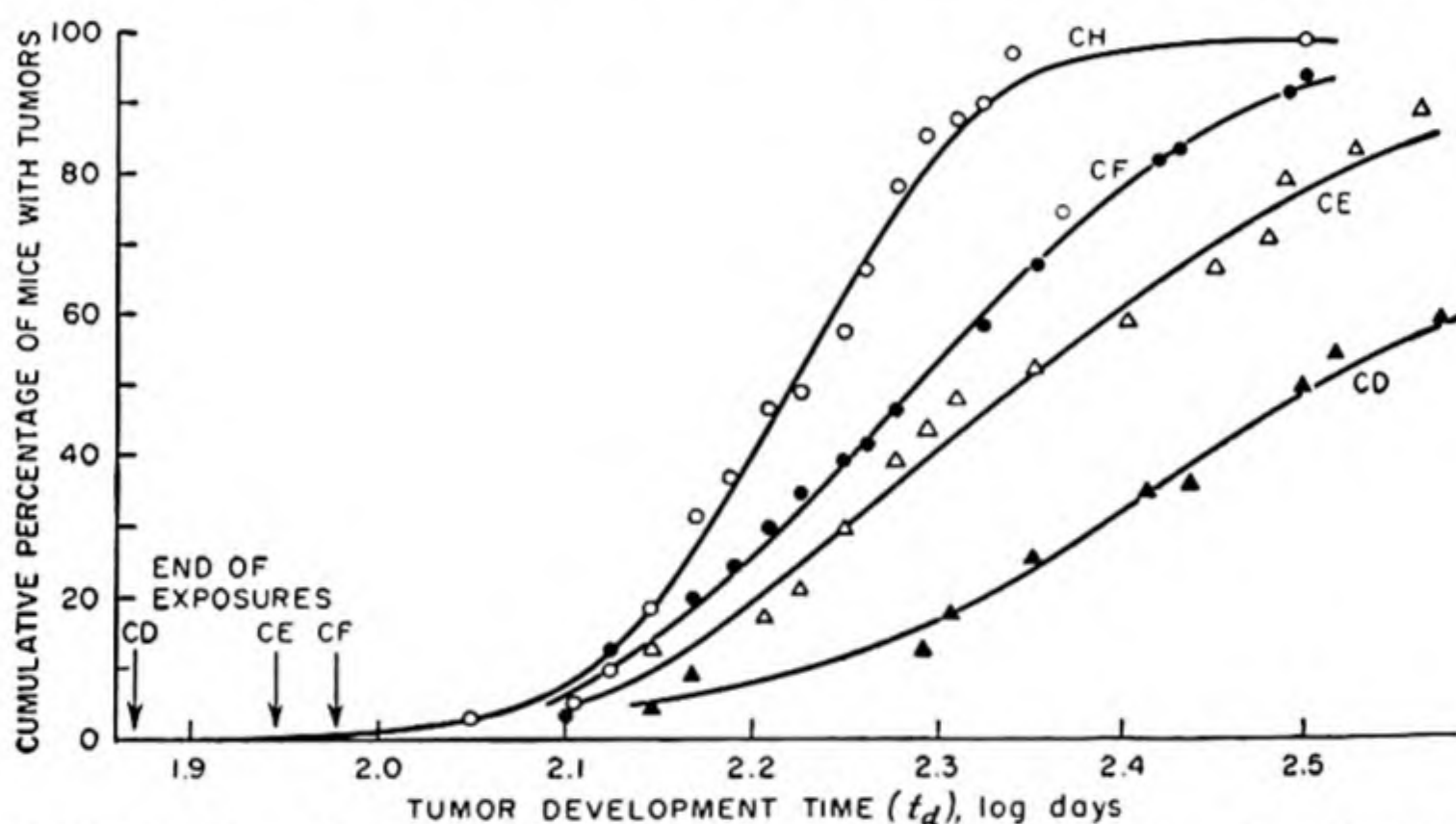


FIG. 14-8. Effect on development time of discontinuation of dose. In series CF, CE, and CD the exposures were discontinued at the times indicated. In series CH the dosage continued until the appearance of tumors. (From Blum, 1950.)

a tumor cannot exceed. Physiological factors, for example, the rate at which materials for growth can be supplied, must set an upper limit. So progressive acceleration of growth, which is the essence of this theory, could be pushed only to a certain point, and, acceleration being directly related to dose, a maximum should be reached beyond which increase in dose would not increase the rate of development. This is what appears to happen. It seems probable that the minimum value of  $t_d$  in the three curves in the figures corresponds to a common maximum rate of proliferation which cannot for physiological reasons be exceeded. Numerically this condition is satisfied.

According to the model illustrated in Fig. 14-5, if the exposures were stopped, a tumor should continue to grow at the rate that was established at the time the exposures ceased. In a certain number of experiments the doses of ultraviolet radiation were stopped before tumors appeared, with a resultant increase in  $t_d$ . This is illustrated in Fig. 14-8, where it is shown that the earlier the doses were discontinued, the longer was  $t_d$ . In Table

TABLE 14-4. EFFECT OF DISCONTINUING SCHEDULE OF DOSES  
(From Blum, 1950b.)

For all experiments, data are for 50 per cent tumor incidence;  $D = 1.8$ ;  $i = 1.4$ . This is the experiment described in Fig. 14-8. All the series were run simultaneously.

Series	$t_1$	$t_2^*$	$t_d$ (observed)	$t_d$ (calculated)
CH.....	166	...	166	164
CF.....	95	100	195	185
CE.....	88	126	214	192
CD.....	74	238	312	212

\* Period without exposure.

14-4, observed values for  $t_d$  are compared with values calculated on the basis of the equations describing progressive acceleration of tumor growth. It is seen that the observed values of  $t_d$  are greater than the calculated, indicating that the rate of tumor growth is slower at some point than is

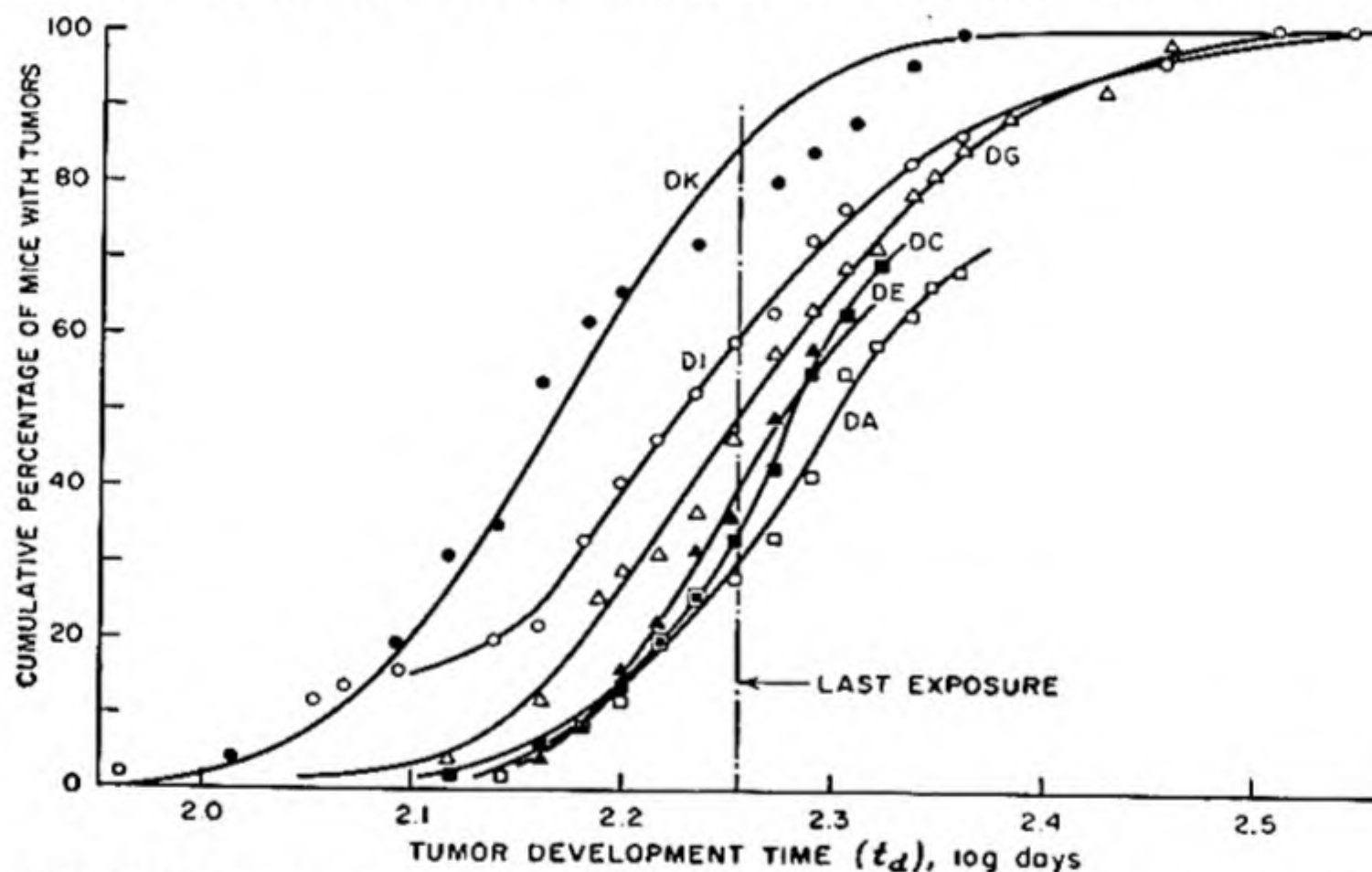


FIG. 14-9. Effect on development time of the interruption of doses. In experiments DA, DC, DE, DG, and DI the exposures were discontinued for 30 days and then resumed. The arrangement of the periods of respite are indicated in Table 14-5. The control, DK, consisted of a smaller number of animals; the curve drawn is based on pooled data. (From Blum, 1950.)

predicted by the equation. The discrepancy between the observed and calculated values of  $t_d$  increases systematically with increase in  $t_d$ .

Another type of experiment is illustrated in Fig. 14-9.<sup>12</sup> In this, an

<sup>12</sup> Similar experiments have been described by Rusch and Kline (1946), but the data are not applicable to the present analysis and therefore direct comparison cannot be made. Their interpretation does not take growth rates into account and is subject to general criticisms already made in this chapter.



initial period of exposures was followed by a rest period of 30 days, after which the exposures were resumed for another period. In some cases there was another terminal rest period, because it became necessary to discontinue the whole experiment. Again the calculated values of  $t_d$  given in Table 14-5 differ somewhat from the observed values, the observed being generally greater than the calculated. The discrepancies seem to be in the same direction as those that result when comparisons of the data for different dosage intervals are considered. All in all, these discrepancies do not seem too important when it is realized that our treatment has been based on a crude model. Figure 14-5, which describes this model, certainly does not accurately describe actual tumor growth; it is hardly to be believed that the growth rate rises immediately on the application of ultraviolet radiation as is indicated in that diagram.

TABLE 14-5. EFFECT OF INTERRUPTING SCHEDULE OF DOSES  
(From Blum, 1950b.)

For all experiments, data are for 50 per cent incidence;  $D = 2.0$ ;  $i = 1.4$ . This is the experiment described in Fig. 14-9. All series were run simultaneously.

Series	$t_1$	$t_2^*$	$t_3$	$t_4^*$	$t_d$ (observed)	$t_d$ (calculated)
DK.....	155	..	...	..	155	155
DI.....	75	30	64	..	169	170
DG.....	47	30	103	..	180	173
DE.....	33	30	117	9	189	176
DC.....	19	30	131	11	191	178
DA.....	5	30	145	20	200	181

\* Periods without exposures.

As regards the more intimate aspects of carcinogenesis by ultraviolet radiation there is relatively little to say. The active hyperplasia which results from sunburn, and which is also very marked in mouse skin subjected to ultraviolet radiation (Grady *et al.*, 1941), attracts attention and suggests that the same or a similar primary mechanism is involved. It is obvious that some acceleration of cell proliferation is involved in cancer development whether or not it is of the progressive type suggested. In this respect it is interesting that there is no real evidence of acceleration of cell division due to the direct effect of ultraviolet radiation on the cell, but only retardation (see Giese, 1947; Blum and Price, 1950). Such evidence does not eliminate the possibility of indirect effects (see Hollaender and Duggar, 1938; Loofbourow and Morgan, 1940).

The idea that a steroid is changed by ultraviolet radiation into a carcinogen was for a time popular. Roffo, who thought cholesterol was the precursor, seems to have been the first to propose this. Various investigators, using a variety of approaches, failed, however, to find evidence to support this idea (see Blum, 1940, for numerous references). Studies on

vitamin D suggest that the activation of the provitamin takes place in the corneum, or at least very superficially (see Blum, 1950a), and it appears that most of the steroids of the skin are found in the corneum rather than in the deeper tissues. Yet the site of carcinogenesis in the case of the mice is deeper than this. There is other evidence against this hypothesis. The polycyclic hydrocarbon carcinogens, which are the type we should expect to find formed under these circumstances, are quite diffusible. If strain A mice are treated with carcinogens either by injection or by painting on the skin, in which case the carcinogen is of course licked off and gets into the alimentary canal, there is a great increase in the incidence of cancers of the lung (Shimkin, 1940). Yet the incidence of lung tumors in this same strain was not found to be greater among mice in which cancer had been induced by ultraviolet radiation from that in untreated controls.<sup>13</sup> Thus it appears that no diffusible carcinogen is formed by the ultraviolet radiation, and this is strong evidence against the participation of a polycyclic hydrocarbon carcinogen in this type of carcinogenesis.<sup>14</sup>

#### THE ROLE OF SUNLIGHT IN CANCER OF THE SKIN OF MAN

After a discussion of the results of animal experiments, the clinical findings bearing on the question of the etiological role of the ultraviolet radiation of sunlight in cancer of the skin of man may be better evaluated. In our introduction four general lines of evidence were mentioned, and these may now be considered.

*Topographical Distribution.* All authorities agree that cutaneous cancer occurs in the white race predominantly on the face; for example, in 91 per cent of the 1626 cases reviewed by Lacassagne (1933) the tumors were on that area. Basal-cell and squamous-cell cancers predominate among skin cancers, and it is these types which display the strong predilection for the face. The other principal site of these tumors is the back of the hand. This evidence alone strongly supports the idea that sunlight, which reaches other areas of the body to a very small extent, has a role in the etiology of basal-cell and squamous-cell cancers. The occasional appearance of one of these tumors on an unexposed area of the body would not negate this conclusion, since no doubt there are other agents which

<sup>13</sup> Actually there was a slight decrease in the treated animals, the probable causes of which have been discussed by Blum (1944). The experiments also indicated a difference in general susceptibility to the development of cancer within the genetically homogeneous strain.

<sup>14</sup> There have been various experiments showing that exposure to ultraviolet radiation decreases the incidence of cancers in mice painted with carcinogenic hydrocarbons. Recently Engelbreth-Holm and Iversen (1947) offered evidence that this is due to photooxidation of the hydrocarbon. This explanation was offered by the writer as early as 1940. Other possible factors may be involved (e.g., Blum, 1943b), but in any event there seems no reason to connect photochemical reactions of these carcinogens with the induction of cancer by ultraviolet radiation.



cause such cancers. We may cite, for example, the kangri cancers resulting from heat, and cancers caused by carcinogenic chemical agents, such as the cancer in chimney sweeps and in workers in crude petroleum. On the other hand, the much more malignant, although fortunately much rarer, malignant melanomas cannot be attributed to the action of sunlight; these tumors parallel in distribution the pigmented nevi (Pack, 1948), and, although a good many melanomas occur on the face, there are other sites of predilection, particularly the genitals and the feet, where sunlight would be least likely to play a part. The melanomas will be excluded for the present discussion; references to cancer of the skin will mean basal-cell or squamous-cell cancers.

There have been attempts to relate the distribution of the cancers of the face to the incidence of sunlight on different areas, but, as pointed out in an earlier paper (Blum, 1940), these are not very convincing. Different writers use different methods of describing the position of the tumors, and this leads to confusion. The analysis of Magnusson (1935) who related the distribution of tumors to the thickness of the epidermis and the consequent penetration of light in these areas is obviously in error because it fails to take into account the factor of diffusion of radiation by the corneum (see Chap. 13). Altogether, such attempts seem to contribute very little support to the argument that cancer of the skin is caused by sunlight. But the basic finding that these tumors are limited in very large proportion to the face is itself sufficient evidence to indict sunlight as a causal factor, particularly when supported by the other lines of evidence which will be discussed. There have been attempts to explain the distribution of cutaneous cancer on other grounds, which do not seem too convincing. Some of these have been summarized earlier (Blum, 1940). Recently Corson *et al.* (1949) have pointed out that tumors may occur in persons wearing spectacles, where these focus the light rays. They attribute this to heating of the skin, but the evidence is not convincing. Some glasses used for spectacle lenses transmit the carcinogenic wave lengths of sunlight to a considerable extent.

*Complexion.* "A commonplace in dermatological lore is that skin cancer occurs more frequently in blonds than in brunets." This statement is found in an article by Taussig and Williams (1940) in which they attempt to characterize skin color and to associate it with cancer of the skin. There have been numerous other attempts of this kind, but on the whole such a correlation is uncertain. It is, of course, very difficult to know what the terms "blond" and "brunet" mean. The idea that pigmented skin is less liable to sunburn than unpigmented skin—the fallacy connected with which was discussed in the last chapter—may have had considerable influence on thinking along this line. Among the white races it is difficult to correlate the threshold for the erythema of sunburn with complexion.

On the other hand, the threshold for sunburn is much higher in Negroes than in the white races. It is, therefore, of interest to find that cancer of the skin is very rare in members of the Negro race in this country (Dorn, 1944), although there seems to be no evidence of a general immunity to cancer in that race. Moreover, it appears that when cancer of the skin does occur in Negroes it has a very different distribution, being located about as frequently on unexposed as on exposed parts (Schrek, 1944a, b). Here is evidence to support the idea that among the white races sunlight is a principal cause of cancer of the skin. Vint (1935) reports a high incidence of squamous-cell cancers among the natives of Kenya, accounting for 36 per cent of the tumors among this Negro population. He points out, however, that these tumors are associated with tropical ulcers of the leg. Basal-cell cancers are rare. This evidence seems to support rather than conflict with the findings on Negroes in the United States, as regards etiology. Roffo (1939) states that in the Argentine all the cases of skin cancer that he observed occurred in foreigners or immigrant families, none in Indians or Negroes.

*Occupation.* It is commonly believed that cancer of the skin is more frequent in outdoor workers than in indoor workers. This is reflected in such terms as "seaman's skin," "peasant's skin," and "farmer's skin," which are applied to allegedly precancerous changes when they occur on the exposed parts. It is obviously difficult to obtain statistics on this. Perhaps the most convincing are those of Peller and Stephenson (1937) and Peller and Souder (1940) who point out that mortality from cancer of the skin and lip is about three times as high for the United States Army and Navy as for the average population for the same age group. Other evidence, some conflicting, has been discussed by the writer elsewhere (1940). All in all, this part of the evidence seems the least conclusive at the present time.

*Distribution.* There have been many statements that the incidence of cutaneous cancer is greater in those regions of the earth which receive most sunlight, but there was for a long time no basis for a critical analysis. In 1944, however, Dorn published extensive statistics which seem to bear out this idea. He examined the incidence of various types of cancer in three groups of urban areas in the United States: (1) Chicago, Detroit, Pittsburgh, and Philadelphia; (2) San Francisco and Alameda County, California, and Denver; (3) Birmingham, Atlanta, New Orleans, Dallas, and Fort Worth. He designates these groups for his purposes as northern, western, and southern, respectively, but actually they form a series of nonoverlapping latitude groups. Their mean latitudes weighted according to populations of the respective areas are  $40^{\circ}$ ,  $38^{\circ}$ , and  $32^{\circ}$ . In Fig. 14-10 Dorn's figures for incidence of cancer are plotted against latitude. A clear-cut north-south distribution of cutaneous cancer incidence is indicated for both sexes of the white populations, with greater incidence in the



south than in the north. In these data the category "cancer of the buccal cavity" includes some tumors of the exposed parts of the lip, which probably accounts for the distinct north-south distribution of the incidence of such tumors. Cancer of all other sites does not show this relation to latitude. A small increase with increasing latitude among males is opposed by a small decrease among females. These statistics suggest that some factor which varies with latitude markedly affects the incidence of cancer of the exposed parts of the body.

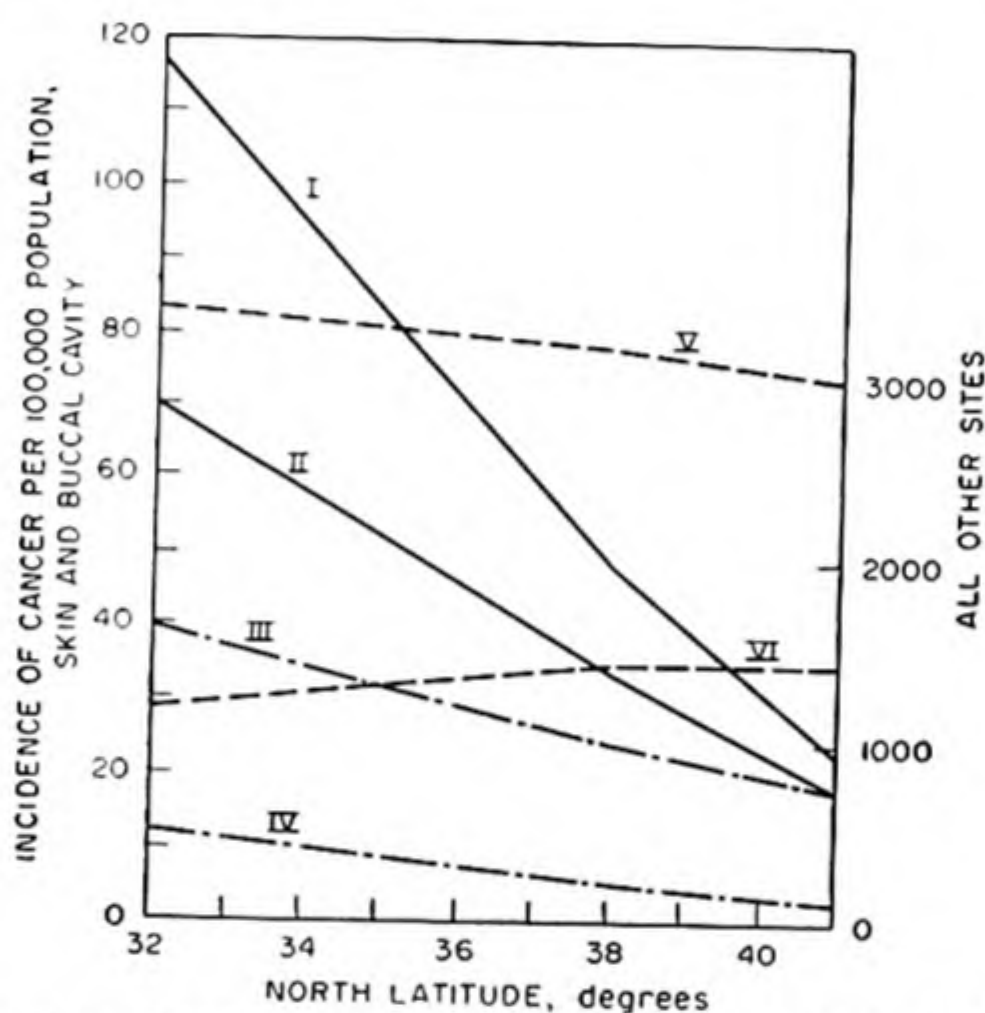


FIG. 14-10. Distribution of cancer with latitude. Curve I, skin, male; II, skin, female; III, buccal cavity, male; IV, buccal cavity, female; V, all other sites, male; VI, all other sites, female. (From Blum, 1948, based on data of H. F. Dorn.)

We may examine the possibility that this factor is sunlight, but to do so we must know the extent of variation with latitude of the particular components of sunlight that we accuse of inducing cancer. The animal experiments indicate that the wave lengths concerned are the same as those that cause sunburn. Total annual sunlight does not vary with latitude to nearly so great an extent as would be required to explain the distribution of cancer incidence shown by Dorn's data; but the carcinogenic wave lengths do show a considerable difference in north-south distribution.<sup>15</sup> In order to make an exact comparison between the incidence of carcinogenic radiation in sunlight at various latitudes and the corresponding incidence of cutaneous cancer, it would be necessary to know the action spectrum of carcinogenesis as well as to have complete data on the shorter wave lengths of sunlight, but it is not feasible to obtain the former

<sup>15</sup> The factors determining the variation of sunlight with latitude are discussed briefly in Chap. 13 and extensively by Sanderson and Hulburt in Chap. 3.

data for reasons that have already been discussed. Even if an action spectrum could be obtained experimentally for the mouse, this would not apply quantitatively to human skin because of the difference in the transmission of the ultraviolet radiation. Direct measurements of the incidence of carcinogenic ultraviolet wave lengths have not been made at a sufficient number of points on the earth to give a complete picture of this distribution in detail, and it would be extremely laborious to do so. Such measurements would, moreover, be of uncertain value in the solution of the present problem, because their interpretation would depend on the missing information regarding wave-length dependence of carcinogenesis in human skin. Some general ideas can be obtained, however, from data that are available.

For the purpose, data calculated by O'Brien (1943) for antirachitic action (based on the absorption spectrum of provitamin D), which has the same long-wave-length limit as carcinogenesis, are used here. There is no reason to believe that the action spectrum for carcinogenesis in man is related to that for antirachitic action, and the action spectrum for the erythema of sunburn might be thought more closely representative. But there are inherent objections to using an accepted erythematous spectrum for this purpose, as was pointed out in Chap. 13; in any case O'Brien's calculations indicate that about the same relative values would be obtained if the erythematous spectrum was used in place of the antirachitic spectrum. Such estimates must obviously be very rough, at best being influenced by numerous factors which cannot be readily taken into account, for example, cloudiness and dust. However, they provide as satisfactory an index as is now available.

The variation of cancer incidence and carcinogenic radiation with latitude is indicated in Fig. 14-11. In order to have a relative basis of comparison, the value for the lowest latitude,  $32^{\circ}$ , has been taken as 100 per cent for each variable. When the data are plotted in this way, the incidence of cutaneous cancer shows about the same magnitude of change with latitude for both sexes (curves III and IV). Two curves for annual incidence for carcinogenic radiation are plotted, one for 2.0 mm of ozone in the atmosphere (curve I) and one for 2.8 mm of ozone (curve II). Neither of these curves shows as great variation with latitude as do the cancer incidence curves. Ozone, which is the principal limiting factor with regard to the shorter wave lengths of sunlight, varies with latitude, and the two curves represent approximate extremes for the latitudes covered by the cancer incidence data, 2.8 mm representing the northernmost and 2.0 mm the southernmost condition. Thus the true range of variation with latitude should be greater than is indicated by either curve alone. In curve V, the annual ultraviolet radiation corresponding to 2.0 mm of ozone is used for latitude  $32^{\circ}$  and that for 2.8 mm of ozone is used for latitude  $41^{\circ}$ . The resulting curve which should represent more



nearly the range of carcinogenic radiation has a slope similar to that of the cancer-incidence curves although not quite so steep. The agreement is certainly as good as might be expected, considering the numerous assumptions that had to be made in order to carry out the analysis. The least that can be said is that the expected variation in the amount of carcinogenic radiation with latitude probably is sufficient to account for the variation of cancer-incidence data with latitude that is indicated by Dorn's data. Such factors as the amount of smoke, cloudiness, and dust

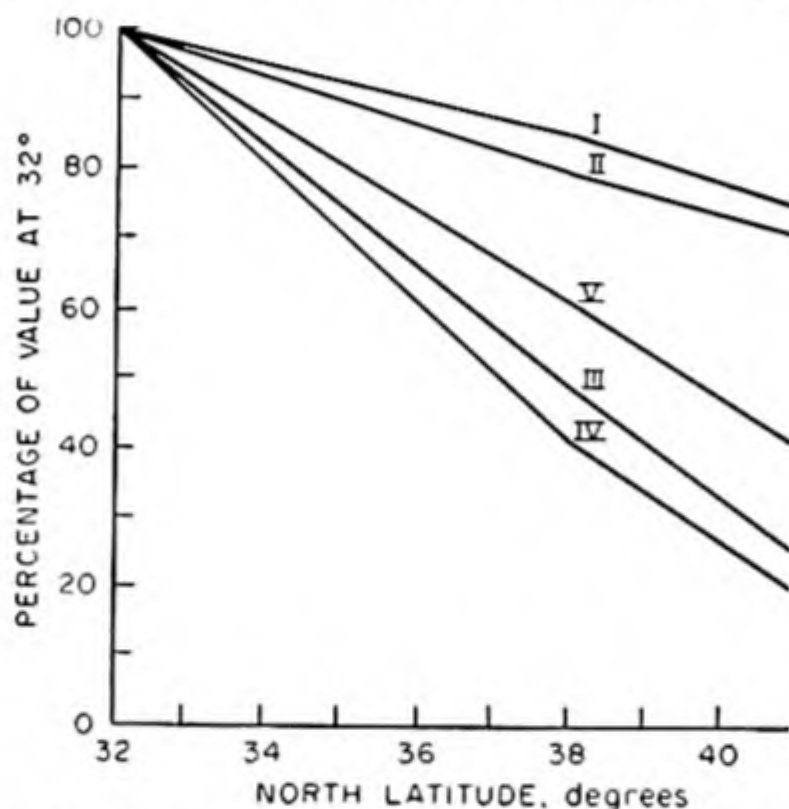


FIG. 14-11. Distribution of carcinogenic radiation and cutaneous cancer with latitude. Curve I, 2.0 mm ozone; II, 2.8 mm ozone; III, incidence of cutaneous cancer, female; IV, incidence of cutaneous cancer, male; V, maximum difference. (From Blum, 1948, based on data of H. F. Dorn and Bryan O'Brien.)

are left out of consideration, and these may limit the amount of carcinogenic radiation in specific areas, but there are no means for estimating their importance.

### PREVENTION

When we consider how the lines of evidence converge, it seems difficult to reach any conclusion other than that the ultraviolet radiation of sunlight is a major causal factor in cancer of the skin in the white population of the United States. The evidence is against applying this conclusion to the Negro population, where the incidence of skin cancer is small in any event.

If such a conclusion is to be accepted even tentatively, the question of possible means of prevention of these cancers arises. Even if it were feasible, it would not be desirable to counsel the complete avoidance of sunlight. This is true particularly since skin cancers of the squamous- and basal-cell types are among the least dangerous of malignant growths, and their incidence is relatively low even under conditions which seem most favorable for their occurrence, for example in the southern United

States. Certainly it is not desirable to instill fear of exposure to sunlight in a large part of the population, but certain prophylactic measures may be suggested. Continuous regular exposures to artificial sources of ultraviolet radiation, even in moderate doses, would seem unwise, particularly when these are used during the winter months to supplement summer exposure, either for cosmetic or therapeutic reasons. Probably a large proportion of the white population could practice such regular exposures without accident, but an unfortunate few might be expected to develop cutaneous cancer. Those individuals who show changes in the skin which the dermatologist recognizes grossly as "precancerous," for instance, the appearance of keratoses, or those who have already had one cancer of the skin of the squamous-cell or basal-cell type, might well be cautioned against even relatively mild continued exposure to ultraviolet radiation. But they should be informed of the character of the carcinogenic radiation and the facts regarding sunlight which have been discussed in the previous chapter. They should know that they are relatively safe in exposing themselves in the early morning or late afternoon, but should avoid the midday sun. They should also be informed that window glass offers good protection against carcinogenic radiation. The use of sunburn preventives when going outdoors may also be recommended, although the uncertainty of evaluating these has already been discussed in the previous chapter.

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